The Role of MeCP2 in Pain and its Regulation by miRNAs

By

Melissa Taft Manners

March 2016

A Dissertation Presented to the Faculty of Drexel University College of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

It is with the deepest gratitude and appreciation that I dedicate this work to my husband, Vincent Manners. He has inspired me and has been my “rock” throughout this process. Additionally, I lovingly dedicate this work to our daughter, Julia, who fills my heart with more joy than I ever imagined was possible, and to our second child whom we are very excited to welcome into the world.

Although animal research is important and necessary for the progression of science, it was always with a heavy heart that their lives were sacrificed in my hands. This thesis is also dedicated to them with respect and gratitude.

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ABSTRACT

The Role of MeCP2 in Pain and Its Regulation by miRNA

Melissa Taft Manners

Seena K. Ajit, Ph.D.

DNA methylation is a dynamic epigenetic modification and this biological signal is read by methyl-CpG-binding protein 2 (MeCP2). MeCP2 regulates gene expression through activation, repression and chromatin remodeling and is crucial for neuronal development and function. Mutations in *MECP2* cause Rett syndrome (RTT) and decreased pain perception is reported in children with RTT, suggesting MeCP2 function is important in modulating pain. The overall objective of this study was to understand the role of MeCP2 in nociception under both physiological and pathological conditions. Dorsal root ganglia (DRG) convey noxious stimuli from the periphery to the central nervous system. Our previous miRNA profiling of DRG in a rodent model for neuropathic pain showed decreased expression of multiple miRNAs predicted to target *Mecp2*. We confirmed MeCP2 upregulation in DRG following nerve injury and repression of MeCP2 by miRNAs *in vitro.* MeCP2 regulates brain derived neurotrophic factor (BDNF) and downregulation of MeCP2 by miRNAs decreased *Bdnf in vitro*. MeCP2 T158A knock-in mice with a mutation in the methyl-CpG binding domain of MeCP2, exhibited reduced mechanical sensitivity. *Mecp2*-null and MeCP2 T158A mice have decreased *Bdnf* in DRG.

MeCP2-mediated regulation of *Bdnf* in the DRG could contribute to altered pain sensitivity.

The functional implication of increased MeCP2 is largely unknown. To identify regions of the genome bound by MeCP2 in the DRG and the changes induced by nerve injury, chromatin immunoprecipitation of MeCP2 followed by sequencing (ChIP-seq) was performed. While the number of binding sites across the genome remained similar in the spared nerve injury (SNI) model and control, SNI induced the redistribution of MeCP2 to transcriptionally relevant regions. To determine how differential binding of MeCP2 can affect gene expression, we investigated mmu-miR-126, a miRNA locus that had enriched MeCP2 binding in the SNI model. Repression of miR-126 and the ensuing downstream molecular changes was confirmed using the SNI model, *Mecp2*-null mice and a neuronal cell line. Our study shows a regulatory role for MeCP2 in that changes in global redistribution can result in direct and indirect modulation of gene expression in the DRG, thereby contributing to epigenetic alterations underlying nerve injury.

CHAPTER ONE: Background and Significance

INTRODUCTION TO PAIN

Nociceptive pain is a protective mechanism to guard against potentially damaging stimuli. However, chronic pain, characterized by pathological nociceptive hypersensitivity [1] is a debilitating condition that affects more than 100 million Americans, costing over $600 billion in healthcare related expenses [2]. Neuropathic pain is triggered by a lesion or disease of the somatosensory nervous system that alters its structure and function [3]. Present treatment options are limited to nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, anticonvulsants, and antidepressants, which together provide pain relief to only about 50% of patients, clearly highlighting the unmet medical need for such a common and widespread ailment [4].

The Pain Pathway

Pain processing begins with nociception, a process where noxious stimuli are detected by peripheral nociceptors. Cell bodies for these nociceptors are located in the dorsal root ganglia (DRG). The DRG has two axonal branches, extending both peripherally and centrally to innervate the peripheral target organ and the dorsal horn of the spinal cord, ultimately conveying sensory information from the periphery to the central nervous system (CNS). The peripheral terminal responds to environmental stimuli including heat, cold, and mechanical stimulation. Both the peripheral and central terminals contain receptors subject to targeting from endogenous molecules. In addition to relaying nociceptive information, both

terminals have directed analgesic therapeutics. For example, opioid receptors at the central terminal are affected by a spinal delivery of morphine, and topical anesthetics block targets at peripheral nociceptors [5]. The DRG are primary sites for pain processing and as such have been the focus of much research to identify molecular targets of pain neurotransmission. Our goal was to determine changes in gene expression and regulation due to peripheral nerve injury, therefore our studies have been conducted primarily in the DRG.

There are two major classes of nociceptors [6]. The first class includes Aδ afferent nociceptors, myelinated medium diameter neurons that convey fast pain and large diameter Aβ fibers that respond to light touch. The second class of nociceptor are the unmyelinated C fibers that convey slow pain and are subdivided based on gene expression properties, into peptidergic (expressing substance P, calcitonin gene related peptide, tyrosine kinase receptor A) and non peptidergic fibers (expressing purinergic receptor $P2X_3$, and Ret receptor for glial cell line-derived family of neurotrophic factors). These classes of nociceptors innervate specific subsections of the dorsal horn of the spinal cord from lamina I to lamina V. Projecting neurons within lamiae I and V are the major output from the dorsal horn to the brain [7]. These neurons form the ascending pathways, including the spinothalamic tract, carrying pain signals to the thalamus which is important for the sensory-discriminative aspects of the pain experience, and the spinoreticularthalamic tract, carrying pain signals to the brainstem. Additional projections to the dorsolateral pons, which communicates with the amygdala is

important for processing information relevant to aversive properties of the pain experience [5].

Animal Models of Pain

There are several rodent models of neuropathic and inflammatory pain generated to investigate the pathophysiology and underlying molecular mechanisms. Inflammatory pain models are usually generated by the injection of an adjuvant (complete Freund's adjuvant) or chemical (carrageenan, formalin, capsaicin, prostaglandin) into the hindpaw, ankle, or knee joint of rodents [8]. Administration of these agents can induce chronic or acute inflammatory pain resulting in reduced pain thresholds.

Peripheral nerve injury models have been developed to better understand chronic neuropathic pain. These models involve nerve injuries to the sciatic or spinal nerve on one side [9]. Nerve injury models involving the sciatic nerve range from a partial or complete transection to ligation or compression. Ligation or transection of spinal nerves also induces neuropathic pain [9]. These models induce neuropathic pain to varying degrees, depending on the severity of the injury. Restriction of injury to one side allows for the stimulation of the injured or uninjured paw in the same animal, thereby enabling discrimination of hypersensitivity in the ipsilateral injured paw induced by the nerve injury.

We employed the spared serve injury (SNI) model of neuropathic pain for our studies. This is a partial denervation model, allowing for investigation of both

injured primary sensory neurons as well as intact sensory neurons [10]. Figure *1A-B* depicts the nerve injury, where the common peroneal nerve and tibial nerve are ligated and sectioned, while the sural nerve remains intact. The model produces severe, chronic neuropathic pain, including allodynia, the experience of pain from a non-painful stimulation of the skin; and hyperalgesia, an abnormally heightened sensitivity to pain. We characterized pain sensitivity by measuring mechanical threshold using von Frey filaments (Figure 1*C*).

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INTRODUCTION TO EPIGENETICS

Epigenetics encompasses heritable alterations in gene expression and chromatin without accompanying changes in the DNA sequence. Epigenetic mechanisms are dynamic and respond to changes in the environment and cellular experiences, resulting in cellular differences in function and provide a mechanism to document history [11]. Epigenetic modifications cause remodeling of chromatin resulting in regulation of gene expression. There are three major mechanisms of epigenetic regulation, including DNA methylation, noncoding RNAs and histone modifications. In this thesis, we focus on miRNA-mediated regulation of gene expression and DNA methylation, specifically methyl-CpG-binding protein 2 (MeCP2), a protein that binds to methylated DNA.

microRNAs

MicroRNAs (miRNAs) are endogenous ~22nt RNAs that regulate gene expression usually by binding to the 3' untranslated region (UTR) of targeting mRNAs for cleavage or translational repression. miRNAs are encoded in various regions of the genome, including introns and exons of both coding and non-coding genes. Promoter regions have not been mapped for most miRNAs. They often have multiple transcriptional start sites [12], and promoters can be distinct or shared with the promoter of the host gene [13]. Following transcription, the primary miRNA undergoes several steps of maturation and processing, nuclear export, and finally the mature miRNA is packaged into RNA-induced silencing complex formation

[14]. miRNAs repress gene expression through two mechanisms; repressing translation and mRNA degradation. mRNA degradation is catalyzed by deadenylating enzymes. Deadenylated mRNAs are decapped and then degraded by exoribonuclease 1. Approximately 60-90% of miRNA-mediated repression occurs through this mechanism in cultured mammalian cells [15]. Partial complementarity of the seed sequence is a determinant in target recognition and is sufficient to trigger translational silencing [16]. In some cases, translation is initiated but miRNA mediated disruption in translation results in a small peptide that is quickly degraded [17].

DNA Methylation

DNA methylation is a chemical addition of a methyl group onto the 5th carbon of cytosine which can directly regulate gene expression. The majority of DNA methylation occurs at CpG sites, referring to cytosine and guanine linked with a phosphodiester bond. Regions of DNA with a particularly high occurrence of CpG sites are called CpG islands [18]. They are defined as regions of more than 200 bases with a CpG content of 50% or greater. About 60% of gene promoters contain CpG sites, and they are usually unmethylated [19]. Methylation at CpG islands is generally associated with gene silencing, and unmethylated CpG islands are associated with gene expression [20]. DNA methylation can directly inhibit transcription by impairing transcription factor binding [21]. CpG sites also occur outside of CpG islands. Recently, it has been shown that DNA methylation can occur outside of the traditional CpG site [22]. In mouse dentate neurons, 75% of

methylation occurred at CpG sites, while 25% of methylation occurred at CpH sites, where H can be adenine, cytosine or thymine.

The broad classification of enzymes involved in DNA methylation are; “writers” to establish methylation patterns, “erasers” to remove methyl groups from nucleotides, and “readers” that recognize, and bind to methylation patterns. DNA methyl transferases (Dnmts) function as “writers” by transferring the methyl group of cosubstrate, S-adenyl methionine, to the fifth carbon of cytosine [21]. Dnmt1 maintains methylation in mitotic cells by recognizing hemimethylated sites and replicating the methylation pattern in the daughter cells. Dnmt3a and Dnmt3b are responsible for *de novo* methylation, modifying the genome in new ways. The role of “eraser” enzymes is to demethylate DNA. Demethylation occurs through a series of enzymatic reactions creating intermediate products of 5-methyl cytosine. The most studied intermediate is 5-hydroxymethylcytosine (5-hmc), which is enriched in active genes in the CNS [23]. This reaction is catalyzed by ten-eleven translocation enzyme and can further oxidize 5-hmc, producing 5-formylcytosine and 5-carboxylcytosine [24]. The base excision repair process removes the oxidized cytosine and replaces it with a non-methylated cytosine [21, 25]. Proteins containing a methyl-CpG binding domain (MBD) “read” DNA methylation patterns. Proteins in this family include MBD1-MBD4 and methyl-CpG-binding protein 2 (MeCP2). These proteins can decipher methylation patterns before binding to methylated DNA, and recently, reader proteins have been found to bind to modified cytosine cites [23, 26].

MeCP2 is a reader protein that has been implicated in pain, and is the focus of this study. We investigated how MeCP2 is regulated by miRNAs and identified regions of the genome that are differentially bound by MeCP2 after peripheral nerve injury.

MeCP2

MeCP2 was the first protein identified containing an MBD. Several additional DNA and protein interacting domains have been characterized (Figure 2). The MBD is the primary region of interaction between MeCP2 protein and DNA, and it enables MeCP2 to specifically bind to methylated CpG sites [27]. Chromatin immunoprecipitation studies have supported this finding by showing preferential MeCP2 binding to methylated regions in the brain, and mutations in this region reduce the affinity of MeCP2 for methylated DNA [28]. This study also quantified the abundance of MeCP2 in the nuclei with levels as high as the histone octamer. This genome-wide binding pattern is interpreted to play a role in dampening transcriptional noise as opposed to regulating expression of individual genes. DNA methylation can occur outside CpG sites, and MeCP2 was shown to bind to these unconventional methylated regions in the brain and in cultured neurons [22]. In cerebellar cells, the MBD also has an affinity, albeit weaker, for 5-hmc and MeCP2 was shown to be a major binding protein for 5-hmc in active genes [23]. This study demonstrated that the R133C mutation occurring in RTT patients within the MBD of MeCP2 inhibits binding to 5-hmc, suggesting that the interaction of MeCP2 with this modified cytosine is important in symptoms associated with RTT.

MeCP2 can also bind to DNA at regions other than the MBD site. Recently, MeCP2 was characterized to contain three AT-hook DNA-binding motif domains [29]. High-mobility group AT-hook family proteins (HMGA) often contain 2-3 AT-hook domains, with a flexible polypeptide linker. This allows multiple contact locations to alter chromatin conformation. Mutations occurring within the AT-hook domain of the C-terminus of MeCP2 disrupt MeCP2 DNA binding, and could further contribute to RTT pathology [29] .

MeCP2 was first identified as a methylation dependent transcriptional repressor. MeCP2 contains a transcriptional repression domain (TRD), and was shown to repress genes with methylated promoters, but not unmethylated promoters [30]. Transcriptional profiling of the hypothalamus from *Mecp2*-null mice showed 15% of affected genes were downregulated in the absence of MeCP2 [31]. Further studies also show that MeCP2 can interact with histone deacetylate (HDAC) complex containing SIN3A, and nuclear receptor co-repressor (NCOR) and silencing mediator of retinoic acid (SMRT), the binding domain of which resides in the TRD of MeCP2 [32]. The significance of MeCP2 interaction with HDAC is further supported pharmacologically, as transcriptional repression is attenuated with an HDAC inhibitor [33]. MeCP2 represses a large number of genes in cultured neurons by preferentially binding to long genes containing methylated CpA sites [34]. Transcriptional profiling of the brain from *Mecp2*-null mice was one of the first studies to demonstrate the activator role of MeCP2 [31]. In these mice, 85% of the genes affected were downregulated in the absence of MeCP2,

suggesting that MeCP2 has a role in activation of these potential targets. Chromatin immunoprecipitation (ChIP) studies confirmed MeCP2 binding to a subset of genes, and immunoprecipitation studies and proteomic analysis revealed that MeCP2 binds to the promoter of activated genes in a complex with the transcription factor, cyclic AMP-responsive element-binding protein 1 (CREB1) [31].

Collectively, these studies demonstrate that MeCP2 binds to methylated DNA in a genome-wide manner, thus affecting a large number of downstream genes. MeCP2 additionally recruits cofactors and can modify chromatin to globally reduce transcriptional noise as well as influence transcription of specific target genes.

EPIGENETIC REGULATION AND PAIN

Molecular and genetic studies have shown a fundamental role for epigenetics in key biological processes and how epigenetic aberrations result in disease. Epigenetic regulation has been explored in the context of peripheral inflammation, plasticity, and cortical pain processing [35]. Nerve injury can induce alterations in chromatin structure and influence molecular and cellular function, which ultimately have the potential to lead to symptoms ranging from allodynia, hyperalgesia, anxiety, and depression [36]. Here we explore alterations in miRNAs and DNA methylation that occur in response to peripheral nerve injury.

miRNAs and Pain

Maintaining appropriate levels of miRNA is very important, as aberrant expression of miRNAs are reported in various diseases, including pathological pain. The role of miRNAs in peripheral pain pathways was globally evaluated using a conditional deletion of Dicer, an enzyme involved in the biogenesis of miRNA, in nociceptors important for inflammatory pain [37]. Deletion of Dicer lead to a broad increase in mRNAs in the DRG. Though these mice had normal acute pain thresholds, inflammatory pain was attenuated, as inflammatory mediators could not enhance the excitability of sensory neurons. Therefore, disruption of miRNA processing in the DRG was sufficient to reduce inflammatory pain [37].

miRNA profiling studies

Surveying tissue and fluids for miRNAs in a disease or animal model can identify how a pathology affects miRNA levels. miRNA profiling conducted in the blood of several animal models of neuropathic, inflammatory, and chemotherapy induced pain resulted in uniquely dysregulated miRNA profiles in each model [38]. Interestingly, all models included dysregulated miRNAs predicted to target factors involved in the Wnt signaling pathway. Studies in DRG sensory neurons following chemotherapy induced pain identified 57 dysregulated miRNAs [39]. Of these differentially expressed miRNAs, inhibition of miR-1a in the DRG alleviated the pain phenotype associated with this model through increased expression of the *Clcn3* chloride channel. We previously determined miRNA alterations in the DRG after peripheral nerve injury, and found differential expression of 63 miRNAs [40]. Table 1 shows a subset of miRNAs downregulated after nerve injury predicted to target MeCP2. Computational target prediction algorithms are commonly used to identify candidate genes targeted by miRNAs. Algorithms are diverse and use different parameters to provide candidate target genes [41]. However, these computational programs use different algorithms and predict different lists of putative targets for a given miRNA [42], yielding both false-positive and false-negative predictions. Nevertheless, prediction tools are immensely valuable in identifying potential targets that can be experimentally validated.

miRNA profiling has also been conducted in various fluids from patients with painful diseases. Patients suffering with fibromyalgia have a low pain threshold.

Compared to healthy controls, 9 miRNAs were downregulated in the cerebral spinal fluid from these patients [43]. Of these miRNAs, miR-145-5p positively correlated with levels of pain and fatigue. Patients with irritable bowel syndrome have higher circulating levels of miR-1250 and miR-342 than healthy controls [44]. These miRNAs are associated with inflammatory pathways and pain signaling. They could serve as biomarkers and additionally, could contribute to the abdominal pain associated with IBS. Patients with complex regional pain syndrome (CRPS) have a unique miRNA signature compared to control subjects, with 18 differentially expressed miRNAs [45]. These miRNA profiles can be useful for both patient stratification and future therapeutic intervention. Ketamine is beneficial in treating CRPS patients, and 33 miRNAs were differentially expressed in responders compared to poor responders in the pre-treatment blood samples [46].

Together, these data demonstrate that pathological pain can affect miRNA expression in tissue and in circulation. With the wide range of downstream targets for each miRNA, large scale effects could ensue from dysregulated miRNAs. Additionally, dysregulated miRNAs have utility as biomarkers, and individual or groups of miRNAs could be considered for therapeutic intervention. In our study, we demonstrate how miRNAs downregulated in the DRG after nerve injury lead to upregulation of target expression and downstream molecular mechanisms.

miRNAs attenuate pain behavior

In addition to pain affecting miRNA expression, miRNAs have been shown to therapeutically attenuate hypersensitivity in animal models of pain. An inflammatory stimulus caused methylation of the miR-219 promoter and reduced miR-126 levels in spinal neurons [47]. This resulted in increased expression of its target, CaMKIIγ. Injection of miR-219 reversed thermal hyperalgesia and mechanical allodynia induced by the inflammatory model. miR-103 was shown to regulate three subunits of Cav1.2-comprising L-type calcium channel in the dorsal horn [48]. Knockdown of miR-103 in rodents resulted in hypersensitivity to pain, while overexpression decreased pain threshold after peripheral nerve injury. miR-146a-5p was shown to be downregulated in a spinal nerve ligation model [49]. Intrathecal injection of miR-146a-5p attenuated mechanical allodynia and decreased expression of TNF receptor associated factor, involved in neuroinflammation. miR-124 is expressed in microglia in the CNS. Intrathecal administration of miR-124 reversed hyperalgesia induced by carrageenan in an inflammatory pain model, and also prevented the development of mechanical allodynia in the SNI model [50]. This study interestingly shows how a single miRNA can both reduce sensitivity induced by inflammatory and neuropathic pain models. Another study of miR-124 showed reduced paw licking, a nociceptive behavior associated with a formalin model of chemical-induced pain, and inhibition of miR-124 further increased nociceptive behavior [51]. These effects were proposed to

be mediated through regulation of MeCP2 and expression of its downstream target, brain derived neurotrophic factor (Bdnf).

These examples demonstrate that miRNA expression is affected by disease and this could have consequences on downstream molecular targets leading to progression or worsening of the disease. The studies mentioned above indicate that administration of miRNAs can attenuate hypersensitivity associated with a pain state and could therefore be considered for therapeutic intervention. In this study, we explore miRNA-mediated regulation by confirming binding and repression of bioinformatically predicted targets. We also employ *in vivo* and *in vitro* overexpression and inhibition studies to validate target expression and investigate consequences on pain behavior.

DNA Methylation and Pain

DNA methylation patterns are important, as aberrant methylation can result in several pathologies involving learning, memory, and neuronal development and function [52, 53]. Studies have also shown alterations in DNA methylation in rodent models of neuropathic pain. A genome-wide methylation study conducted in the DRG 24 hours after peripheral nerve injury reported global remodeling of DNA methylation [54]. Differentially methylated CpG sites were found in promoters, exons, and introns occurring at thousands of CpG sites independent of CpG islands. Affected genes were associated with neurobiologically relevant mechanisms in the peripheral nervous system and nerve injury. Differentially

methylated CpG sites within genic regions showed hypermethylation of 82% and hypomethylation of 18% of affected genes. Genes with hypermethylated promoters were shown to be both upregulated and downregulated. A recent study investigating methylation changes induced by chronic pain 9 months after SNI surgery observed over 12,000 methylated gene promoters in the prefrontal cortex. Increased methylation was also observed in circulating T-cells [55]. In the lumbar spinal cord, a chronic constriction injury model of neuropathic pain caused an increase in global DNA methylation levels 14 days after surgery, and an upregulation of MeCP2 [56]. This increase in methylation and MeCP2 expression was partially prevented with intrathecal administration of a DNA methyltransferase inhibitor, 5-azacytidine (5-AZA). A global methylation study conducted in the brain 6 months after SNI surgery found decreased global methylation in the amygdala and prefrontal cortex [57]. Interestingly, environmental enrichment reversed the abnormal global methylation in the prefontal cortex in SNI model mice. Nociceptive thresholds were positively correlated with global methylation.

Therapeutic potential DNA methylation

An important aspect of epigenetics, is the dynamic nature of these mechanisms, and therefore they are inducible as well as reversible. Epigenetic reprogramming has recently been demonstrated, by converting differentiated cells into pluripotent states [58]. Since epigenetic marks are labile, reprogramming using pharmacological modulators could provide an avenue for therapeutic intervention [59]. This has been demonstrated with DNMT inhibitors, 5-azacytidine and

decitabine, currently approved for clinical use in cancer [60, 61], and is being extended to other therapeutic areas. It was shown that the chronic constriction injury (CCI) model induced a global increase in DNA methylation levels in the spinal cord [56]. Administration of 5-azacytidine not only reduced methylation levels, but alleviated both the mechanical allodynia and thermal hyperalgesia associated with this model. Another study demonstrated that an intraperitoneal injection of DNMT inhibitor, 5-azacytidine-2'-deoxycytidine reduced incision-induced nociceptive behavior [62]. Mechanical allodynia, thermal hyperalgesia, and paw swelling associated with hindpaw incision were alleviated, suggesting both an analgesic and anti-inflammatory effect. Therapeutics targeting epigenetic mechanisms thus holds a promising future. However, these mechanisms are global and therefore lack of drug selectivity and side effects can potentially have large repercussions.

MeCP2 and Pain

Abnormal sensory thresholds observed in RTT patients initially linked MeCP2 to pain [63]. Studies investigating acute pain threshold of mice mimicking MeCP2 mutations observed in patients show reduced pain sensitivity [64-67]. Additionally, expression levels of MeCP2 are affected in nerve injury models and inflammatory pain models. Together, the RTT patients and animal models implicate that regulated levels of MeCP2 are important for normal pain thresholds.

Rett Syndrome and pain

Mutations in *MECP2*, an X-linked gene, can cause RTT, an autism spectrum disorder leading to severe physical and neurological disabilities. Onset of symptoms occur after 6 to 18 months of age and include developmental regression, loss of hand skills with gain of stereotypical hand movements, impaired mobility, loss of speech, and respiratory disorders [68]. In addition, decreased pain perception is often reported by the caretakers of children with RTT. Clinical case studies include generally impaired nociception [69], and a patient after corrective surgery for scoliosis required very little postoperative analgesic compared to healthy children who had the same surgery [70]. A study evaluating the response of parents to questionnaires from the Australian Rett Syndrome Database and the International Rett Syndrome Phenotype database, assessed the prevalence of aberrant pain sensitivity of RTT patients [63]. In the total population study, the prevalence of an abnormal pain response was over 75%, with 86% of those patients having a decreased response to painful stimulus. Patients were also stratified based on 12 different genomic mutations in *MECP2*, and in each subgroup the majority of patients presented with decreased pain sensitivity.

MeCP2 expression in pain

MeCP2 expression is influenced by pain. In a time course evaluation of MeCP2 expression after SNI surgery, MeCP2 was found to be downregulated 10 days post-surgery in the DRG, and upregulated in the dorsal horn after 7 days.

These effects seem to be temporal, as we evaluated expression of MeCP2 in the DRG and dorsal horn 4 weeks post SNI surgery and found upregulation in the DRG [64] and downregulation in the dorsal horn (Figure 3), which was also observed by others [71]. MeCP2 is expressed in nociceptive and non-nociceptive neurons in the DRG [67], and neurons, oligodendrocytes and astrocytes in the dorsal horn [71]. After SNI surgery, MeCP2 expression is decreased in damaged neurons of the dorsal horn [71]. MeCP2 expression levels were also modulated by inflammatory pain as expression was increased in the dorsal horn 7 days after inflammatory stimulus [71]. Inflammatory stimulus increased phosphorylation of MeCP2 in lamina I projection neurons in the dorsal horn, which are essential for the development of pain [72]. This resulted in the dissociation of MeCP2 from the genome, thereby alleviating repression of genes linked to pain in projection neurons. The dynamic expression of MeCP2 in the dorsal horn and the DRG after nerve injury suggests that nerve injury and inflammation induces expressional changes in MeCP2.

Central mechanisms of pain and morphine seeking behavior are also linked to MeCP2. MeCP2 directly represses the transcriptional repressor, histone dimethyltransferase G9a, resulting in increased Bdnf [73]. This mechanism contributes to the regulation of the emotional response to pain and opioid reward. MeCP2 mediated repression of the glutamate AMPA receptor subunit, Glua1, contributes to the morphine-seeking behavior associated with morphine withdrawal administered after pain [74]. Importantly, these studies demonstrate

that nerve injury and self-administration models can result in the dynamic binding of MeCP2 to genes and promoters thereby directly and indirectly affecting gene expression.

Thus, the expression levels of MeCP2 as well as the dynamic binding of MeCP2 to the genome contribute to pain sensitivity. Further studies are needed to understand how expression changes in the DRG lead to alterations in gene expression and the progression of pain pathology.

Mouse models of RTT

Over 600 unique mutations resulting in RTT occur in the gene encoding *MECP2*, with 9 mutations accounting for over 75% of cases [75]. There are several animal models created to recapitulate the human condition, ranging from point mutations to complete *Mecp2* knock out. Mice harboring these genetic mutations closely mimic symptoms found in humans.

The pain thresholds of several *Mecp2* mutant mouse lines have been evaluated. A mouse line generated by disrupting the 3'UTR of MeCP2 resulted in a 50% reduction in transcript and protein levels of MeCP2 [65]. This mutation resulted in phenotypic abnormalities consistent with RTT. In addition to the many behavioral alterations associated with RTT, they observed increased threshold in the hot plate test, indicating reduced thermal sensitivity compared to wild type littermates. Another study conducted by the same group assessed female *Mecp2*$^{-/+}$ mice in two different background strains [66]. The thermal sensory

thresholds of these mice were significantly increased in the hot plate test, again indicating decreased pain sensitivity. Overexpression of MeCP2 [76] increased mechanical and thermal pain threshold [67]. These studies show that the reduced pain sensitivity observed in RTT patients is reproduced in mouse models. Both increased and decreased MeCP2 levels contribute to reduced pain sensitivity. Collectively, these data suggests an important role for MeCP2 in nociception, and disruption of MeCP2 expression could alter pain phenotype.

In our study we used mouse models of RTT for molecular characterization and for assessment of pain behavior. The *Mecp2*-null mouse line was generated by deleting all but the amino-terminal 8 amino acids of MeCP2 [77]. MeCP2 is an X-linked gene, therefore females are heterozygote $Mecp2^{-/+}$ mice and males are hemizygote $Mecp2^{-/y}$ mice, with no detectable levels of MeCP2 transcripts or protein. Mice also develop a severe RTT phenotype with a delayed onset of symptoms. Thus, these mice serve as an excellent model to study molecular alterations that occur in the absence of MeCP2 in the DRG. Female mice are very important for studying the phenotype associated with RTT. However, we used male mice, as our goal was to assess the role of MeCP2 in pain and male hemizygotes are complete MeCP2 knock outs. Male mice die at a young age and have symptoms that make evaluation of sensitivity unreliable, therefore, we did not conduct a behavioral assessment of these mice.

For both molecular and behavioral characterization, we used the MeCP2 T158A knock in mouse [78], which recapitulates one of the most common

mutations associated with RTT. The point mutation in threonine 158 results in the conversion to either methionine or alanine and disrupts the MBD. Over 70% of patients with this mutation have decreased pain sensitivity [63]. These mice develop RTT like phenotypes, similar to those observed in *Mecp2*-null mice, including age-dependent developmental regression, motor dysfunction, and learning and memory deficits. This mutation leads to decreased MeCP2 expression, and increased expression of targets normally repressed by MeCP2. ChIP results indicated reduced binding to methylated loci, and reduced affinity for methylated DNA. Therefore, the MeCP2 T158A mouse serves as an excellent model to assess how a disruption in the methyl binding domain of MeCP2 affects pain sensitivity, and ensuing molecular effects in the DRG. We used male *Mecp2*$^{T158A/y}$ hemizygotes for our studies.

REGULATION OF MECP2 BY MIRNAS

Regulation of MeCP2 is of great interest as aberrant expression of MeCP2 leads to neurological disorders. MeCP2 is subjected to regulation by miRNAs through confirmed and predicted binding sites within the 8kb 3' UTR. Further exploring the transcriptional degradation and translational repression of MeCP2 by miRNAs in the context of pain is a focus of our study.

Several miRNAs have been confirmed to regulate MeCP2 through transcriptional degradation or translational repression. miR-132 overexpression in cultured cortical neurons decreased MeCP2 expression levels, and also lead to reduced expression of the MeCP2 target, Bdnf [79]. Both Bdnf and miR-132 levels are decreased in *Mecp2*-null mice compared to wild type littermates, indicating that MeCP2 activates expression of miR-132. Additionally, inducing phosphorylation of cAMP response element-binding protein (CREB) increased miR-132 levels and decreased MeCP2 expression. Together, *in vivo* and *in vitro* studies demonstrate the homeostatic regulation of MeCP2 by miR-132.

MeCP2 represses expression of miR-212 in the dorsal striatum, and this regulation leads to reduced cocaine self-administration in rats [80]. Conversely, overexpression of miR-212 repressed MeCP2 expression, and the inhibition of endogenous miR-212 increased MeCP2 *in vitro*. Overexpression of miR-212 in the dorsal striatum reduced MeCP2 levels and cocaine intake in a cocaine self-administration model conducted in rats. This relationship indicates a negative

feedback state, where MeCP2 inhibits miR-212, and the opposite relationship is also true in the dorsal striatum.

Patients with Beckwith-Wiedemann syndrome, a disease characterized by overgrowth present at birth and increased risk of cancer, have imprinting defects that lead to over expression of miR-483-5p. Fibroblasts from these patients contain high levels of miR-483-5p and decreased levels of MeCP2 [81]. MeCP2 overexpressing cells have an abnormal dendritic spine phenotype and miR-483-5p mediated repression of MeCP2 in these cells rescued the phenotype.

Formalin injection into the hindpaw induces inflammatory pain, and this results in downregulation of miR-124a in the spinal cord [51]. miR-124a was shown to reduce expression levels of MeCP2, and its target Bdnf. Injection of miR-124a reduced the pain phenotype associated with this model, while inhibitors of endogenous miR-124a increased pain behavior.

These studies demonstrate the importance of precise control of MeCP2 and regulatory miRNAs and their potential therapeutic role. Although the regulatory role of the above miRNAs has been confirmed, there are many other miRNAs predicted to target MeCP2. Our study investigates miRNA-mediated regulation of MeCP2 focusing on a subset of miRNAs that are dysregulated in pain.

OBJECTIVES AND RESEARCH OVERVIEW

Mutations in *MECP2* cause RTT, and decreased pain sensitivity is commonly observed in RTT patients, suggesting normal MeCP2 function is important in modulating pain [63]. An earlier study in our laboratory identified 63 miRNAs that were differentially expressed in the DRG from a rodent model of neuropathic pain [40], including 12 downregulated miRNAs bioinformatically predicted to interact with the MeCP2 3'UTR, listed in Table 1 [41]. Modulation of MeCP2 has been observed in the DRG and spinal cord from rodent models of pain [71, 82]. Our studies of DRG from the SNI model of neuropathic pain showed an increase in MeCP2 expression. The combination of downregulation of miRNAs predicted to target MeCP2 and increased MeCP2 expression in DRG suggests that modulation of MeCP2 could be miRNA-mediated. We hypothesize that the downregulation of miRNAs induced by SNI contributes to increased MeCP2 expression in the DRG. This increase in MeCP2 and alterations in its binding can lead to dysregulation of gene expression (Figure 4). These concepts are explored through two specific aims, as described below.

Aim 1: Determine whether miRNAs downregulated in neuropathic pain can directly modulate MeCP2 expression. To determine if a subset of miRNAs downregulated in neuropathic pain can regulate MeCP2 expression, we used reporter assays and confirmed binding of miR-132, miR-301 and miR-19a to the MeCP2 3'UTR. Overexpression of these miRNAs in Neuro 2a cells decreased MeCP2 expression, while inhibitors increased expression. Furthermore, MeCP2 is

a transcriptional regulator of BDNF, and miRNA mediated repression of MeCP2 resulted in its downregulation. BDNF is upregulated in DRG neurons from neuropathic pain models [83, 84]. We found that MeCP2 T158A mice have reduced pain sensitivity, similar to the RTT phenotype, and also have reduced Bdnf transcripts in the DRG. Confirmation of MeCP2 regulation of Bdnf in the DRG and disruption of this system in pain provide a mechanistic link for MeCP2 expression in mediating pain.

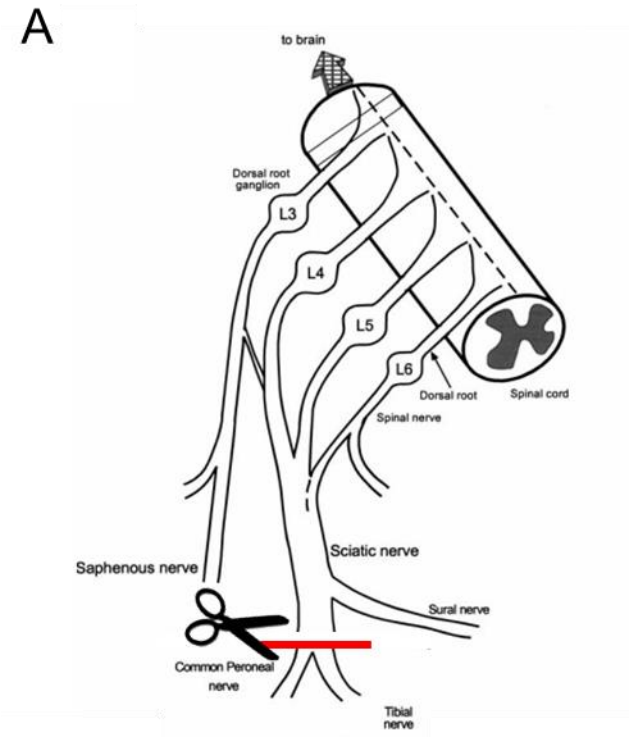
Aim 2: Determine differential binding of MeCP2 to the genome in an SNI model and investigate resulting changes in gene expression. To determine genes that were differentially bound by MeCP2 in the SNI model, we conducted chromatin immunoprecipitation sequencing (ChIP-seq). Comparative analysis of ChIP-seq results from SNI and sham controls identified a redistribution of MeCP2 to transcriptionally relevant regions and non-coding RNAs. Further analysis revealed an enrichment of MeCP2 binding to the miR-126 locus, resulting in repression of miR-126. We investigated the expression levels of miR-126 and its targets in the DRG from SNI model mice as well as *Mecp2*-null mice to determine expression changes in the absence of MeCP2. We further explored the effect of miR-126 overexpression *in vitro* and *in vivo* by measuring target expression and effects on pain behavior in the SNI model.

CHAPTER 1: Figure legends and Figures

Figure 1: SNI model of neuropathic pain

The SNI model was used in this study to induce neuropathic pain from peripheral nerve injury ***A*****,** Partial deneravation of the sciatic nerve, including the common peroneal and tibial nerve, while the sural nerve remains intact. ***B*****,** Nociception can be measured using the lateral portion of the hind paw, which is innervated by the sural nerve. ***C*****,** von Frey filaments were used to measure the mechanical threshold of mice after SNI or sham surgery. Figure adapted from I. Decosterd, C.J. Woolf. Pain 87 (2000) 149-158.

Figure 1


A
to brain
Dorsal root ganglion
L3
L4
L5
L6
Dorsal root
Spinal cord
Spinal nerve
Saphenous nerve
Sciatic nerve
Sural nerve
Common Peroneal nerve
Tibial nerve
B
Lateral
Medial
Sural
Tibial
Saphenous

C

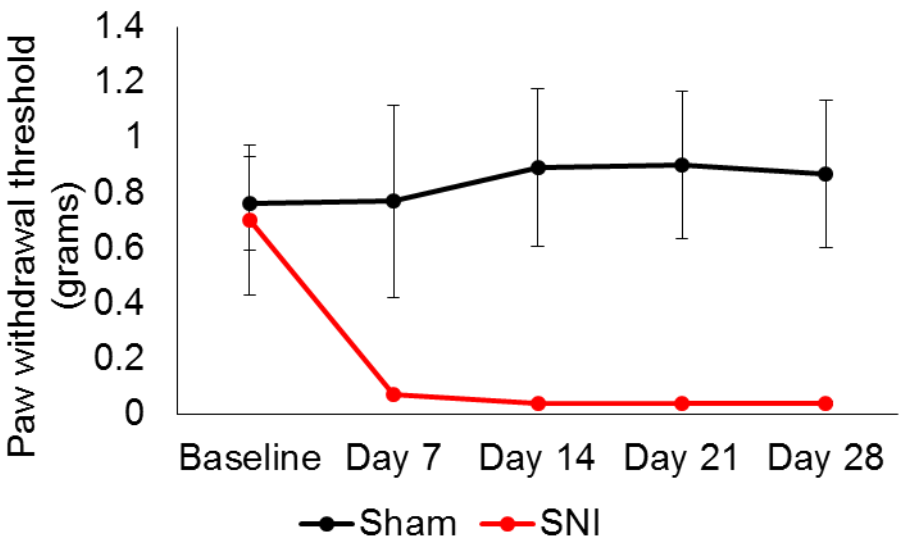

Paw withdrawal threshold (grams)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Baseline
Day 7
Day 14
Day 21
Day 28
Sham
SNI

Figure 2: Domains of MeCP2

MeCP2 has several DNA binding domains and protein binding domains. MBD: Methyl CpG binding domain, TRD: Transcriptional repression domain, NID: NCOR-SMRT interaction domain (NCOR= nuclear receptor co-repressor, SMRT =silencing mediator of retinoic acid), H1-H3: AT hook domains

Figure 2

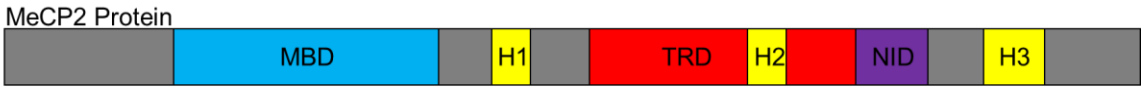


Figure 3: Expression of MeCP2 in the dorsal horn after SNI surgery

It was previously reported that MeCP2 expression is decreased in the dorsal horn following SNI surgery. We measured expression levels 4 weeks after surgery. ***A*****,** Mecp2 mRNA levels were not significantly decreased the SNI model after peripheral nerve injury. B**,** MeCP2 protein was significantly decreased after SNI surgery. Significance determined using Student's *t*-test *p* value *** < 0.001.

Figure 3

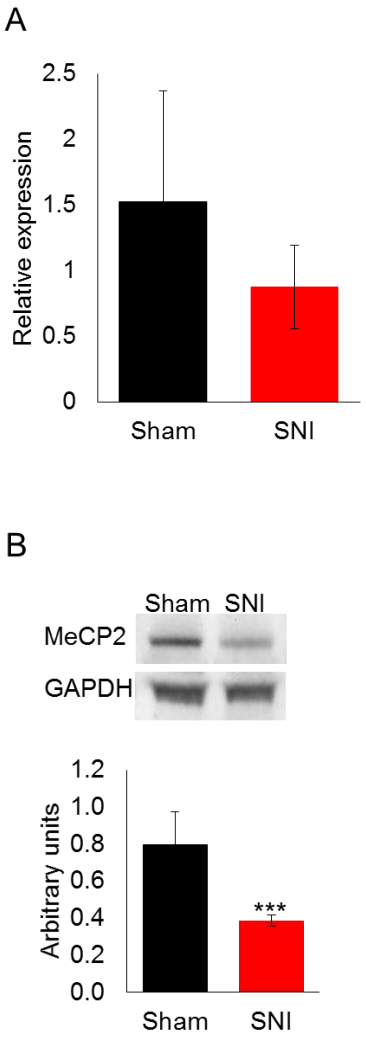

A
2.5
2
1.5
1
0.5
0
Relative expression
Sham
SNI
B
Sham
SNI
MeCP2
GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Arbitrary units
***
Sham
SNI

Figure 4: Schematic of our hypothesis; molecular changes occurring in the DRG after nerve injury

After peripheral nerve injury, miRNAs that are predicted to repress MeCP2 expression are downregulated, while MeCP2 protein is upregulated. MeCP2 can both activate and repress gene expression, and we predict that increased expression could lead to changes in gene regulation. Therefore, we hypothesize that increased MeCP2 expression can both induce upregulation and downregulation of genes linked to pain.

Figure 4

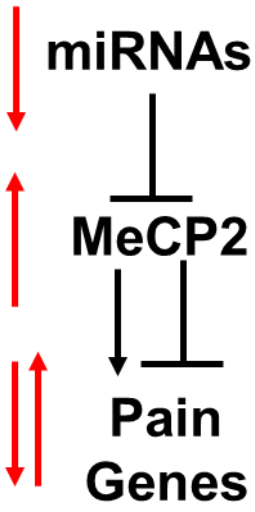


Table 1: Significantly downregulated miRNAs following SNL surgery predicted to target the 3'UTR of MeCP2

miRNA profiling was conducted on rodent DRG after peripheral nerve injury. Of the 63 miRNAs altered, 12 miRNAs predicted to target MeCP2 were decreased in the L4 and L5 ipsilateral DRG compared with the contralateral DRG. These miRNAs were derived based on the filters of the t-test p value <0.01. The table was sorted by fold change for the SNL L4. Minus sign indicates downregulation. miRNAs selected for experimental validation are highlighted in yellow.

Table 1

miRNA	SNL-L4 fold change	SNL-L4 P value	SNL-L5 fold change	SNL-L5 P value
hsa-miR-132	-234.12	5.97E-05	-2.36	0.037041
hsa-miR-20a	-177.70	0.0034	-1.20	0.052
hsa-miR-301	-45.30	0.000016	-1.80	0.00029
hsa-miR-133a	-24.56	0.000378	-10.62	1.75E-05
hsa-miR-17-3p	-16.20	0.0024	-3.60	0.16
hsa-miR-148b	-15.64	1.88E-05	-2.07	0.001357
hsa-miR-148a	-8.51	5.91E-06	-3.03	9.5E-06
hsa-miR-30a-5p	-5.88	4.07E-06	-1.28	0.008209
hsa-miR-30d	-5.30	1.62E-06	6.53	0.407083
hsa-miR-19a	-4.10	2.9E-06	1.70	0.0012
hsa-miR-181b	-3.70	0.00029	-2.00	6.3E-06
hsa-miR-299-5p	-2.60	0.0029	-1.30	0.042

CHAPTER TWO: miRNA Mediated Regulation of MeCP2 in the DRG after Nerve Injury

ABSTRACT

Nerve injury induces chronic pain and dysregulation of microRNAs in the dorsal root ganglia (DRG). Several downregulated microRNAs are predicted to target methyl-CpG-binding protein 2 (MeCP2) transcript. *MECP2* mutations cause Rett syndrome (RTT) and these patients report decreased pain perception. We confirmed MeCP2 upregulation in DRG following nerve injury and repression of MeCP2 by miRNAs *in vitro.* MeCP2 regulates brain derived neurotrophic factor (BDNF) and downregulation of MeCP2 by microRNAs decreased *Bdnf in vitro*. MeCP2 T158A mice exhibited reduced mechanical sensitivity and *Mecp2*-null and MeCP2 T158A mice have decreased *Bdnf* in DRG. MeCP2-mediated regulation of *Bdnf* in the DRG could contribute to altered pain sensitivity.

INTRODUCTION

Chronic pain is a prevalent, disabling health condition affecting more than 100 million people in the United States [85]. Neuropathic pain caused by injury or dysfunction of the nervous system can result in peripheral and central sensitization, a state of hyperexcitability due to reduction in threshold and an amplification in the responsiveness of nociceptors [86]. Elucidation of signaling pathways underlying pain hypersensitivity is crucial in improving our understanding of molecular mechanisms that drive plasticity in the nervous system promoting the development and maintenance of chronic pain states [5]. Animal models of neuropathic pain have been pivotal in the exploration of molecular mechanisms of pain underlying nerve injury [87]. Our miRNA profiling study in the DRG from a nerve injury model identified 63 miRNAs differentially regulated compared to sham controls [40]. Bioinformatic prediction indicates 12 downregulated miRNAs are predicted to target the 3' untranslated region (UTR) of *Mecp2*. Translational repression of MeCP2 mediated by binding of miRNAs to the 3'UTR has been reported [51, 79, 81, 88, 89].

MeCP2 binds methylated DNA, and together with co-repressors or transcriptional activators, mediates downstream changes in gene expression either directly or by altering chromatin structure [31, 90]. Mutations in the X-linked *MECP2* gene cause RTT [91, 92]. Decreased pain perception is commonly reported in children with RTT [63, 93]. The abnormal sensitivity can be as high as 75%, with a potential relationship between hyposensitivity and specific mutations

in the *MECP2* gene [63]. The observations from RTT patients indicate that decreased functional MeCP2 contributes either directly or indirectly to reduced pain sensitivity. Since reduced pain sensitivity observed in RTT patients results from a decrease in functional MeCP2 protein, we postulated that a decrease in miRNAs that bind and repress translation of MeCP2 will cause an increase in MeCP2 levels and thus contribute to hypersensitivity.

MeCP2 can mediate downstream transcriptional changes of a large number of genes and depending on its interacting protein partners and target genes, MeCP2 can act either as an activator or repressor [31, 94]. MeCP2 is a known regulator of *Bdnf* [95], a neurotrophin important in both neuronal development and peripheral pain mechanisms [96]. BDNF levels in the hypothalamus correlated with MeCP2 levels, with lower levels in *Mecp2*-null mice and higher levels in *MECP2* overexpressing mice compared to controls [31]. The role of BDNF in both inflammatory and neuropathic pain is well established [84, 97-99]. Here we investigated if miRNAs downregulated in a rodent model of neuropathic pain that modulate MeCP2 expression can induce changes in *Bdnf* levels in Neuro 2a cells.

Several mouse models have been generated for investigating MeCP2 function [100], including alteration of the endogenous *Mecp2* gene, or introduction of the human *MECP2* gene with RTT-associated mutations. One of the most common mutations observed in RTT is in amino acid T158, located at the C terminus of the methyl-CpG binding domain of MeCP2. It has been reported that 70.6% of patients with this mutation have decreased pain sensitivity [63]. The

phenotype of MeCP2 T158A knockin (KI) mice resembles developmental symptoms found in RTT patients [78]. MeCP2 T158A mice showed a reduction in MeCP2 binding to methylated DNA and a decrease in MeCP2 protein stability. Female $Mecp2^{+/-}$ mice with a conditional mouse allele that expresses 50% of the wild-type level of MeCP2 had a slower reaction to a conductive heat stimulus [65, 66]. We sought to assess the pain sensitivity of MeCP2 T158A mice as well as expression of *Bdnf* to test our hypothesis that MeCP2 plays a role in mediating pain sensitivity and confirm the functional implication of a mutation in the methyl binding domain.

MATERIALS AND METHODS

Cell culture, transfection and luciferase reporter assay

HEK293 and Neuro 2a cells obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO2. For the reporter assay HEK293 cells were co-transfected with precursor miRNA or anti-miRNA plasmid (GeneCopoeia) and luciferase reporter plasmid containing the 3'UTR of *Mecp2* using Lipofectamine 2000 (Life Technologies) for 48 hours. The ~8.5kb 3'UTR sequence of *Mecp2* was cloned downstream of the luciferase reporter gene as 4 constructs of ~2kb fragment each (GeneCopoeia). The fragment with the miRNA target sequence of interest (1-2210bp or 6360-8591bp) was co-transfected with corresponding miRNA. The Luc-Pair Duo-Luciferase assay (GeneCopoeia) was used to measure firefly and renilla luciferase according to the manufacturer's instructions. Firefly luciferase measurements normalized to renilla was used as a transfection control. For monitoring changes in endogenous MeCP2, Neuro 2a cells were transfected with precursor miRNA or anti-miRNA plasmid (GeneCopoeia) using X-tremeGENE HP (Roche-biochem) for 48 hours.

Western blot

Protein from Neuro 2a cells or DRG was isolated using radioimmunoprecipitation assay buffer (Thermo Scientific). For western blotting, 20µg protein lysates were resolved by a 4-12% SDS–PAGE gel, transferred to the nitrocellulose membrane.

The membranes were probed with MeCP2 antibody [78] at 1:3000 dilution overnight. Chemiluminescence was detected using FluorChem M System (Protein Simple). The membrane was also probed with goat anti-rabbit GAPDH-HRP (1:2000 dilution, Santa Cruz) as a loading control. Quantification was determined using UN-SCAN-IT software, MeCP2 expression was normalized to GAPDH.

Immunocytochemistry

Neuro 2a cells grown on 12mm glass coverslips were transfected with miRNA precursor plasmids using X-tremeGENE HP DNA transfection reagent for 48 hours. Cells were fixed in 4% formaldehyde and blocked in 10% NGS before a 3 hour incubation in 1:200 anti-MeCP2 antibody (mentioned above). Anti-Rabbit-IgG Atto 647N secondary antibody (Sigma) was used for detection of MeCP2. Coverslips were mounted onto slides using Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were acquired using the 60x objective on the Olympus 1X81 confocal microscope and Fluoview FV10-ASW software. Image J was used for quantification, MeCP2 levels were normalized to DAPI staining of the nucleus, then, transfected (GFP positive) and untransfected (GFP negative) cells were compared.

Quantitative RT-PCR

RNA was purified from Neuro 2a cells, DRG collected from SNI, sham control, *Mecp2*-null, MeCP2 T158A and the corresponding wild-type littermate mice using the mirVana RNA isolation kit (Life technologies). cDNA synthesis and qRT-PCR

were performed as previously described [101]. The Assay ID for the Taqman primer probes used were Mm01193535_M1 (*Mecp2*) and Mm04230607_s1 (*Bdnf*). *Gapdh* was used as the normalizer and a *t*-test was used to perform statistical analysis.

Animal model of neuropathic pain

The care and use of all mice were approved by the Institutional Animal Care & Use Committee of Drexel University College of Medicine. The SNI model was generated using 8-week old C57BL/6 male mice (Taconic) as previously described [10, 102]. Briefly, mice were anesthetized with isoflurane during surgery. The common peroneal and tibial nerves of the left paw were ligated, 2-4mm of the nerve was sectioned and removed distal to ligation. Sham mice underwent the same surgical procedures as the SNI group without ligation and sectioning. Development of mechanical hypersensitivity was assessed using von Frey filaments [102] and the Mann-Whitney U test was used to calculate significance. L4 and L5 DRG on the ipsilateral side of surgery were collected 4 weeks post SNI surgery at 12 weeks of age.

Behavior studies using MeCP2 T158A mice

Male MeCP2 T158A mice and wild-type littermates were purchased from Jackson laboratories (Bar Harbor, Maine). Thermal and mechanical sensitivity were determined using the Hargreaves' method and von Frey filaments respectively.

Behavior testing and tissue collection was conducted at 12 weeks of age. Mann-Whitney U test was used for statistical analysis.

RESULTS

Chronic neuropathic pain induced MeCP2 upregulation in DRG

MeCP2 has been predicted to play a role in pain and modulation of MeCP2 has been observed in various inflammatory and neuropathic pain models [82, 103]. To investigate whether MeCP2 expression is altered in a neuropathic pain state, we generated an SNI model. We confirmed increased mechanical sensitivity and data 4 weeks post-surgery on 12 week old mice is shown in Figure 1*A*. Though there was a trend, the increase in Mecp2 mRNA in DRG after nerve injury was not significant (Figure 1*B*). However, there was a significant increase in MeCP2 protein in the SNI model (Figure 1*C*). This increased expression of MeCP2 in a neuropathic pain state could in part be mediated by the reduced expression of miRNAs predicted to target Mecp2 in the DRG [40]. We therefore sought to determine the regulatory role of a selected subset of miRNAs on MeCP2 expression.

Confirmation of miRNAs predicted to target Mecp2 3'UTR

To experimentally validate the bioinformatics prediction of miRNAs targeting the *Mecp2* 3'UTR, a luciferase reporter assay was performed. We tested a subset of miRNAs that were decreased in the DRG from a nerve injury model based on seed sequence complementarity, varying positions throughout the 3'UTR, the presence of multiple binding sites, and those not predicted to target *Bdnf* mRNA. Reduction in luciferase expression and therefore, miRNA binding to the *Mecp2* 3'UTR was

validated for miR-19a, miR-301 and miR-132 (Figure 2*A*), while miR-17 and miR-181 did not reduce luciferase activity (data not shown).

MeCP2 expression is modulated by miRNAs

To further evaluate regulation of MeCP2 expression from miRNA binding, Neuro 2a cells were transfected with precursor miRNAs or antagomirs that are inhibitors of endogenous miRNAs. Our data indicate that transfection of Neuro 2a cells with miR-19a, miR-301 and miR-132 did not decrease *Mecp2* mRNA (Figure 3*A*) but reduced MeCP2 protein as shown in western blot (Figure 3*B*) and immunocytochemistry of MeCP2 in cell transfected with individual miRNAs (Figure 3*D-F*). The antagomirs of miR-301 and miR-132 increased MeCP2 expression (Figure 3*C*). We did not observe an increase in MeCP2 protein after the transfection of miR-19a inhibitor which could be due to a low endogenous level of miR-19a in Neuro 2a cells, the inhibition of which would not lead to the modulation of MeCP2 expression. Since we did not observe significant changes in *Mecp2* mRNA after over expressing miRNAs, we conclude that *Mecp2* regulation by miRNAs tested is mediated by translational repression and not through RNA degradation.

miRNA-mediated decrease of MeCP2 lead to concomitant reduction in Bdnf

To determine if miRNA mediated regulation of MeCP2 affects expression of the MeCP2 target genes, we measured *Bdnf* mRNA after transfecting Neuro 2a cells with miR-19a, miR-301 and miR-132. Figure 4*A* shows that there was a reduction

in *Bdnf* transcripts after overexpressing miRNAs. Additionally, inhibiting endogenous miR-132 and miR-301 resulted in increased *Bdnf* transcript (Figure 4*B*). The miRNAs used in this study were confirmed to target the *Mecp2* 3'UTR, but are not predicted to bind the *Bdnf* 3'UTR. Thus, the reduced expression of *Bdnf* can be a direct consequence of miRNA mediated regulation of MeCP2.

MeCP2 T158A mice have reduced mechanical sensitivity

Mice expressing 50% of the wild-type level of MeCP2 and female $Mecp2^{+/-}$ mice have reduced thermal sensitivity [65, 66]. We wanted to further investigate the effect of MeCP2 mutations on pain behavior by assessing nociception in MeCP2 T158A mice. We observed that MeCP2 T158A mice have an age dependent reduction in mechanical sensitivity compared to wild-type littermate control mice (Figure 5*A*). Although thermal sensitivity changes have been reported in MeCP2 mutant mice [65, 66], we found no significant changes in thermal sensitivity as determined by Hargreaves' method between MeCP2 T158A and wild-type littermate control mice (Figure 5*B*). These results indicate a role for MeCP2 in mediating mechanical sensitivity.

Bdnf is downregulated in the DRG of *Mecp2*-null mice and MeCP2 T158A mice

Several studies using mouse models have shown a direct correlation between MeCP2 and *Bdnf* expression in the brain indicating that MeCP2 regulates *Bdnf* [95]. We examined *Bdnf* expression in DRG from *Mecp2*-null mice. Figure 6*A*

shows a reduction in *Bdnf* mRNA levels in *Mecp2*-null mice compared to control. Thus MeCP2 mediated regulation of *Bdnf* expression in DRG appears to be similar to that observed in brain. We then investigated *Bdnf* levels in DRG from MeCP2 T158A mice to determine if a loss of function mutation would yield a similar result (Figure 6*B*). We observed a decrease in *Bdnf* mRNA in the DRG of MeCP2 T158A mice as was previously reported in the brain [78]. These results indicate that in DRG, the role of MeCP2 as an activator is important for precise regulation of *Bdnf* and alteration of *Bdnf* in DRG may be a contributing factor leading to aberrant pain sensitivity in RTT.

Bdnf is decreased in MeCP2 T158A SNI model compared to wild-type SNI model mice

To determine how changes in MeCP2 in the DRG affect pain behavior, we generated an SNI model with MeCP2 T158A mice and wild-type littermate controls. Unfortunately, conducting mechanical and thermal sensitivity assessment post SNI surgery was not possible in the MeCP2 T158A mouse; post-surgical over grooming behavior resulted in further physical disability compared to wild-type littermates. We collected the DRG from MeCP2 T158A mice 4-weeks post SNI and from wild-type SNI mice, and measured *Bdnf* transcripts. After nerve injury, MeCP2 T158A mice have decreased *Bdnf* compared to wild-type littermate control mice (Figure 6*C*). This suggests that in the absence of fully functional MeCP2, the upregulation of *Bdnf* is impaired even after nerve injury, again suggestion a regulatory role of MeCP2 on *Bdnf.*

DISCUSSION

The role of MeCP2 in the development and function of the central nervous system is well established and neuronal dysfunction contributes to symptoms associated with RTT [66, 92]. Previous studies investigating the role of MeCP2 in pain have shown differences in expression pattern of MeCP2 in DRG and spinal cord from various rodent models of pain. An increase in MeCP2 has been associated with the development of neuropathic pain in the chronic constriction injury model in rats 14 days after injury [56]. MeCP2 is known to play an important role in signaling in the superficial dorsal horn upon induction of peripheral inflammation by injecting complete Freund's adjuvant (CFA) in rat ankle joint. This study showed that phosphorylation of MeCP2 led to the release of DNA-bound MeCP2, relieving the repression on a few MeCP2 target genes in lamina I neurons. These genes were upregulated rapidly in this model of inflammatory pain [72]. MeCP2 expression was increased in the superficial dorsal horn 7 days following CFA injection in the ankle joint but decreased 7 days following SNI [71]. This study also showed that MeCP2 levels were decreased in activating transcription factor 3 (ATF3)-positive neurons in DRG after SNI [71]. Another report showed an increase in *Mecp2* transcripts in the spinal cord 2 hours after peripheral injection of formalin [51]. Upregulation of MeCP2 in the central nucleus of the amygdala was reported in the CFA model [73]. Differences in models, tissues, and time points can influence gene expression and so we performed qPCR and western blot analysis for MeCP2 using DRG from a

mouse SNI model of neuropathic pain 4 weeks after surgery. We observed an increase of MeCP2 protein in DRG.

MeCP2 can epigenetically regulate miRNAs [104] and dysregulation of miRNAs was observed in the cerebella of *Mecp2*-null mice [105]. miRNA-mediated alteration of MeCP2 was reported to alter pain threshold in wild-type mice. miR-124a was downregulated in spinal cord after peripheral noxious stimulation with formalin and this was associated with an increase in *Mecp2* mRNA [51]. Intravenous administration of a miRNA-124a inhibitor enhanced the nociceptive behavior and miRNA-124a mimic significantly reduced formalin-induced inflammatory pain. In addition to confirming that *Mecp2* is a target for miR-124a, this study showed administration of miR-124a mimic induced a decrease in *Mecp2* mRNA and miR-124a inhibitor enhanced MeCP2 levels in the spinal cord [51]. In another study, intrathecal miR-124 treatment reversed persistent hyperalgesia induced by carrageenan and prevented the development of mechanical allodynia in the SNI model of chronic neuropathic pain [50]. Thus future studies investigating the role of miRNAs validated to target *Mecp2* will be beneficial in confirming their role in pain.

Our previous study investigating miRNA changes in DRG identified several miRNAs predicted to target *Mecp2* 3'UTR. The miRNAs were downregulated after nerve injury and because of the expected inverse correlation in expression between miRNAs and genes they regulate, we selected a subset of 5 miRNAs for further studies. Of these, miR-19a, miR-301 and miR-132 were confirmed to bind

and regulate *Mecp2* by translational repression. miR-132 is a previously validated miRNA for MeCP2 [79] and we included it as a positive control. This reduction of MeCP2 protein resulted in decreased *Bdnf*, indicating that activation by MeCP2 is an important regulatory mechanism for normal expression of BDNF. Our findings that inhibition of miRNA-mediated repression increased MeCP2 and *Bdnf* is similar to what was reported for miR-132 in cultured rat neurons [79].

Alterations in BDNF expression is a common phenomenon in both RTT and pain states, albeit in reverse direction. BDNF is an important modulator of sensory neurotransmission in nociceptive pathways both at spinal and supraspinal levels and has an important role in the development of central sensitization that underlies many forms of hyperalgesia [96]. Many of the biological effects of BDNF are mediated by the high affinity postsynaptic receptor Tropomyosin receptor kinase B (TrkB). TrkB signaling contributes to both the induction and maintenance of injury induced persistent pain [106] and Trk inhibitors have been developed for the treatment of pain [107]. In addition to correlation of BDNF levels in the hypothalamus with MeCP2 levels, with lower levels in *Mecp2*-null mice and higher levels in *MECP2* overexpressing mice compared to controls [31], disruption of total BDNF levels and secretion in *Mecp2*-null neurons indicate that functional MeCP2 is important for BDNF expression [108]. BDNF-related therapies have shown a reversal of cardiorespiratory deficits in *Mecp2*-null mice and is being pursued for the amelioration of RTT-like neurological symptoms in mouse models [95]. Our qPCR studies showed that *Bdnf* levels in the DRG were downregulated in both

Mecp2-null and MeCP2 T158A mice. Thus the reduction in endogenous *Bdnf* could be one of the contributing factors leading to decreased pain sensitivity. Furthermore, after SNI surgery, MeCP2 T158A mice maintained lower *Bdnf* levels than wild-type mice. This indicates that increased Bdnf in the DRG after nerve injury is in part mediated by MeCP2. A recent study investigating the role of MeCP2 in pain regulation and morphine reward showed that MeCP2 and histone dimethyltransferase G9a induce a transcriptional de-repression of *Bdnf* in central nucleus of the amygdala, promoting pain behavior through BDNF upregulation [73]. Thus in addition to direct activation of *Bdnf*, MeCP2 in its role as a transcriptional repressor of G9a, further enhance BDNF levels in chronic pain.

Our studies showed that mice with the loss of function mutation, T158, have reduced mechanical sensitivity compared to wild-type littermates. Previous studies have shown that different MeCP2 mutant mouse lines, including $Mecp2^{Flox/y}$ mice expressing 50% of the wild-type level of MeCP2 and female $Mecp2^{+/-}$ mice have reduced thermal sensitivity [65, 66] but the mechanical sensitivity was not investigated in these mutant mice. Our assessment of thermally induced nociception showed no differences in MeCP2 T158A mice and wild-type littermates. This could be due to the specific mutation of MeCP2, or the methodology used to assess thermal sensitivity. Since BDNF release in dorsal horn in neuropathic pain model is associated with thermal hyperalgesia, [109, 110], we postulate that other modes of action may be playing a role in mediating mechanical sensitivity in T158A mice. Studies investigating function of DRG

neurons in naïve rodents have shown that delivering BDNF directly to DRGs induced mechanical allodynia, conversely, after nerve injury direct administration of anti-BDNF antibody to injured DRG attenuated mechanical allodynia [111]. MeCP2 and BDNF are expressed in microglia and astrocytes in addition to neurons [112] and additional studies are needed to determine the contribution of these different cell types in modulating aberrant pain sensation in MeCP2 T158A mice. A recent study investigated the role of miR-183, a miRNA that was significantly downregulated in the ipsilateral L5 DRG after SNL [113]. Intrathecal administration of miR-183 attenuated mechanical allodynia and this was associated with the downregulation of BDNF transcripts in DRG [113]. This study demonstrated that downregulation of BDNF in DRG was correlated with attenuated mechanical allodynia in SNL model of neuropathic pain. Our data shows that mutations in *Mecp2* can reduce mechanical pain sensitivity. Since MeCP2 can mediate downstream transcriptional changes of a large number of genes, we acknowledge the likely contribution of other dysregulated genes to aberrant pain sensitivity in MeCP2 T158A mice. Additional studies are needed to assess how altered gene expression resulting from the weaker DNA binding properties of MeCP2 with the T158A mutation [78] can influence mechanical but not thermal sensitivity in the T158A KI mice.

In conclusion, our results indicate that miRNA-mediated regulation of MeCP2 can contribute to the mechanical hypersensitivity observed in neuropathic pain models by altering BDNF. Neuropathic pain induces MeCP2 and BDNF

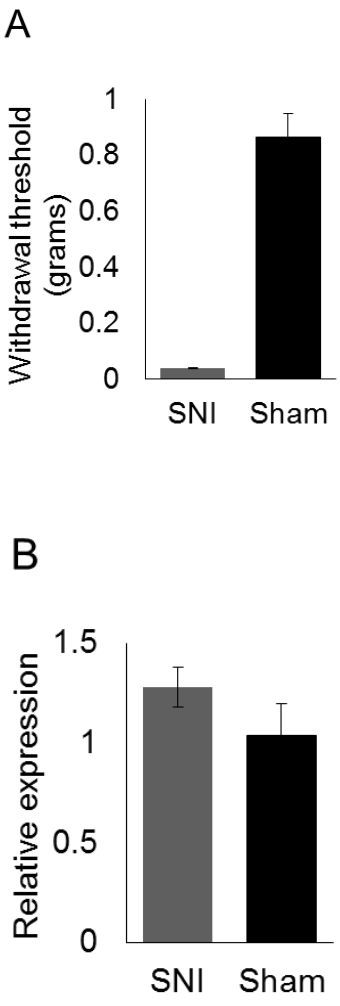
expression in the DRG; conversely, RTT mouse models lacking or expressing reduced levels of MeCP2 have decreased *Bdnf* that can contribute to mechanical hyposensitivity. Further investigations including *in vivo* delivery of miRNAs targeting *Mecp2* followed by characterization of pain behavior and investigation of MeCP2 expression and downstream targets in naïve mice, RTT and pain models can provide insights on therapeutic options for both pain and RTT.

CHAPTER 2: Figure legends and Figures

Figure 1: MeCP2 expression in DRG 4 weeks after SNI or sham surgery

A**,** von Frey filaments used to assess paw withdrawal threshold confirmed that mice were hypersensitive 4 weeks post SNI surgery (n=10). Significance was determined using Mann-Whitney U test *p value* <0.001. ***B*****,** Taqman analysis of *Mecp2* mRNA showed a trend toward increased expression that was not significant in SNI model compared to sham control (n=7). *Gapdh* was used as the normalizer. ***C*****,** Western blot analysis of MeCP2 expression in DRG showed a significant increase in SNI model, determined using Student's *t*-test, *p* value *<0.05. Tissue from 9 animals was pooled into 3 samples (n=3). Protein expression was normalized to GAPDH.

Figure 1


A
Withdrawal threshold (grams)
1
0.8
0.6
0.4
0.2
0
SNI
Sham
B
Relative expression
1.5
1
0.5
0
SNI
Sham

C

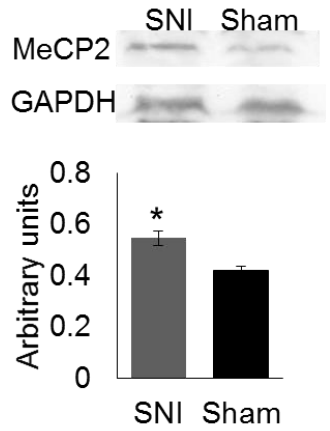


Figure 2: miRNA binding to MeCP2 3'UTR

Luciferase assay showing miR-19a, miR-301 and miR-132 binding to the 3'UTR of *Mecp2*. The luciferase activity was measured 24 hours after miRNA transfection. The data expressed as percentage of control is the average of 3 independent experiments. Statistically significant difference from control was calculated using one way ANOVA and Student's *t*-test, *p* value **<0.01, ***<0.001.

Figure 2

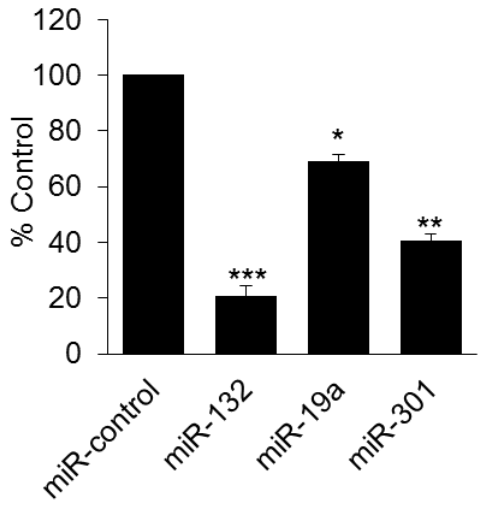
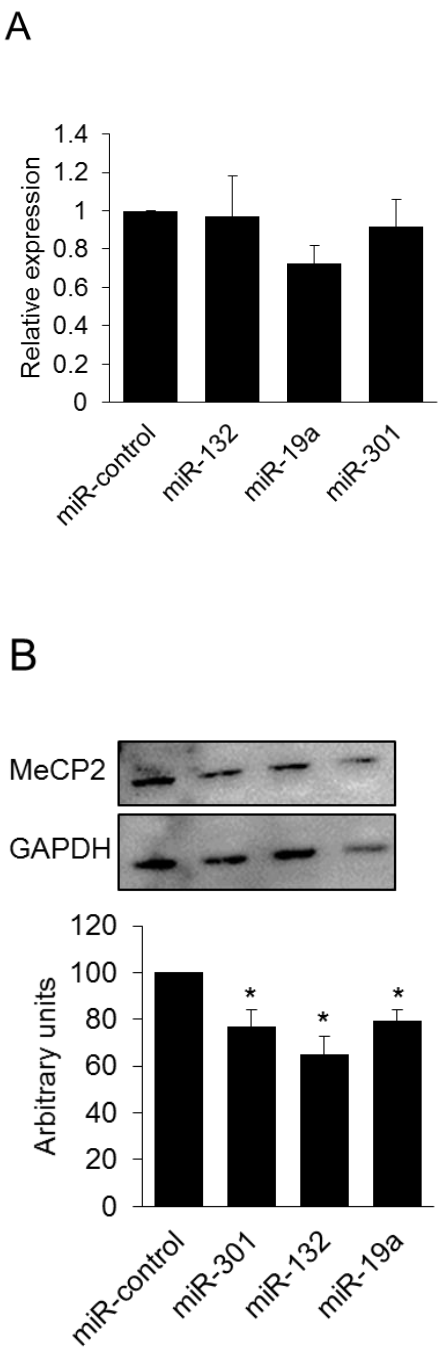

120
100
80
60
40
20
0
% Control
*
**
***
miR-control
miR-132
miR-19a
miR-301

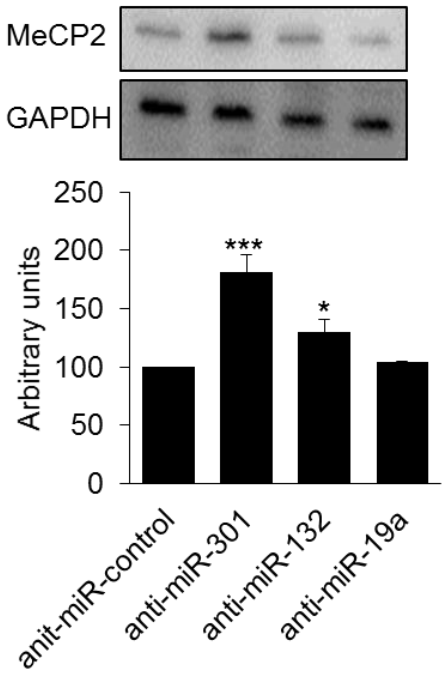
Figure 3: Regulation of MeCP2 by miRNAs

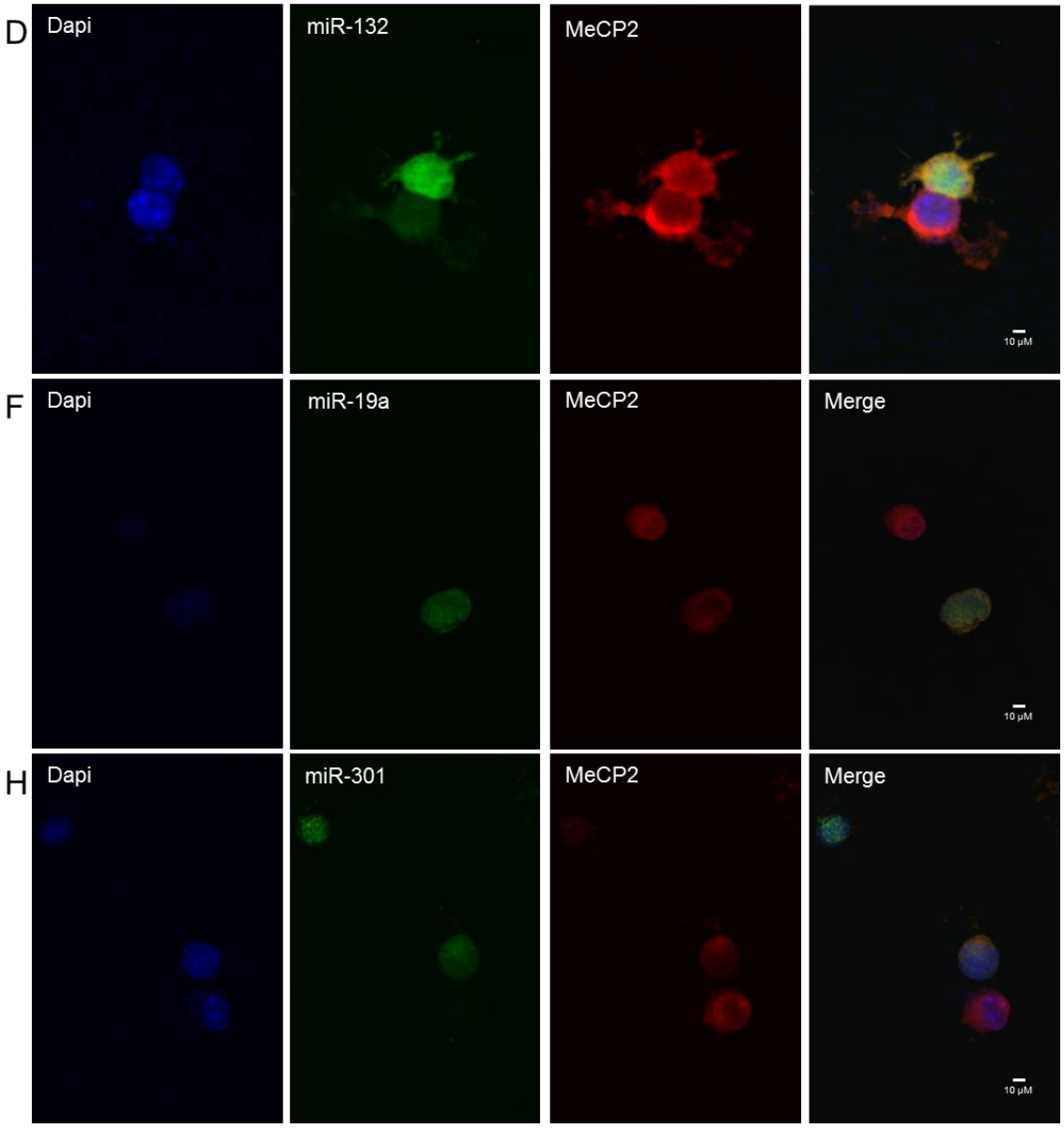
A**,** Taqman analysis of *Mecp2* in Neuro 2a cells 48 hours after miRNA transfection showed no significant reduction in mRNA (n=3). ***B*****,** Western blot analysis after transfection of Neuro 2a cells with miR-19a, miR-301 and miR-132 significantly reduced MeCP2 protein (n=3). ***C*****,** MeCP2 expression increased when cells were transfected with anti-miR (miRNA inhibitors) for miR-301 and miR-132; (n=3, representative blot shown). ***D,E*****,** Immunocytochemisty for MeCP2 in Neuro 2a cells transfected with miR-132. MeCP2 levels were normalized to DAPI staining of the nucleus and graphically represented as expression relative to untransfected cells. Representative images are shown, n=3. ***F,G*****,** Immunocytochemistry and quantification for miR-19a transfected cells. ***H,I,*** Immunocytochemistry and quantification for miR-301 transfected cells. Significance was determined using Student's *t*-test, *p* value *<0.05, **<0.001 ***<0.001.

Figure 3


A
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
miR-control
miR-132
miR-19a
miR-301
B
MeCP2
GAPDH
Arbitrary units
120
100
80
60
40
20
0
*
*
*
miR-control
miR-301
miR-132
miR-19a

C


MeCP2
GAPDH
250
200
150
100
50
0
Arbitrary units
***
*
anit-miR-control
anti-miR-301
anti-miR-132
anti-miR-19a


D
Dapi
miR-132
MeCP2
10 µM
F
Dapi
miR-19a
MeCP2
Merge
10 µM
H
Dapi
miR-301
MeCP2
Merge
10 µM

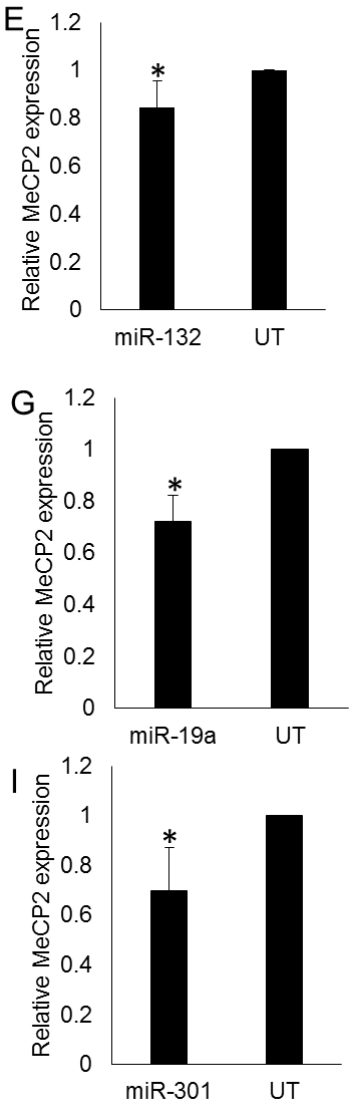

E
Relative MeCP2 expression
1.2
1
0.8
0.6
0.4
0.2
0
*
miR-132
UT
G
Relative MeCP2 expression
1.2
1
0.8
0.6
0.4
0.2
0
*
miR-19a
UT
I
Relative MeCP2 expression
1.2
1
0.8
0.6
0.4
0.2
0
*
miR-301
UT

Figure 4: MeCP2 mediated changes in *Bdnf* transcripts.

A, miRNA-mediated reduction of MeCP2 expression decreases *Bdnf* mRNA in Neuro 2a cells 48 hours after miRNA transfection as determined by Taqman analysis, and ***B*****,** Antagomirs, inhibitors of endogenous miRNAs, increase *Bdnf* mRNA. *Gapdh* was used as the normalizer, n=3. Significance determined using Student's *t*-test *p* value *<0.05, **<0.001.

Figure 4

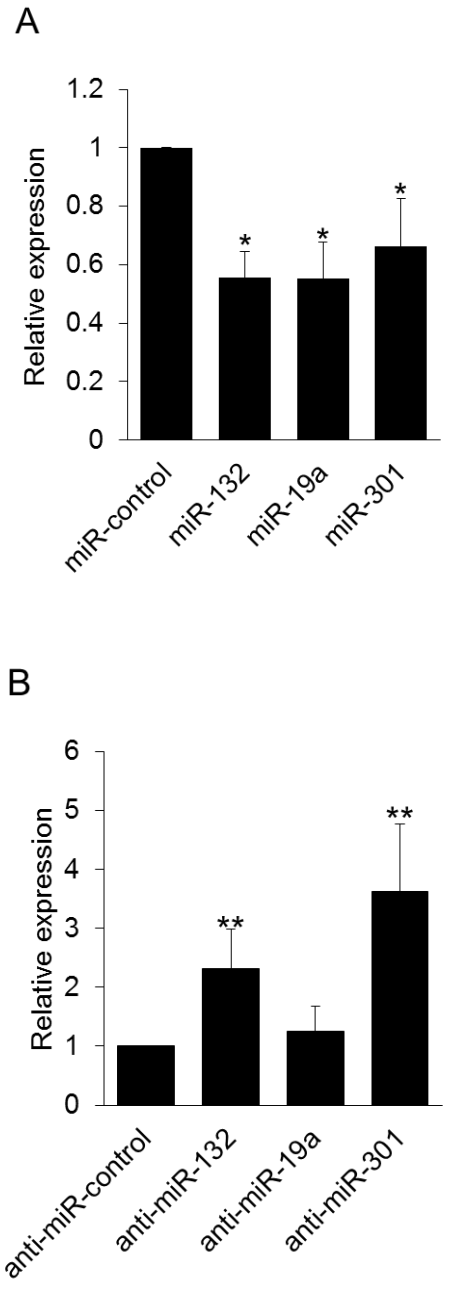

A
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
miR-control
miR-132
miR-19a
miR-301
B
Relative expression
6
5
4
3
2
1
0
**
**
anti-miR-control
anti-miR-132
anti-miR-19a
anti-miR-301

Figure 5: MeCP2 T158A knock in mice have decreased mechanical withdrawal threshold.

A, Mechanical sensitivity measured by von Frey filaments showed an increase in withdrawal threshold in MeCP2 T158A KI mice, indicating hyposensitivity compared to wild-type littermate controls. ***B,*** Thermal sensitivity was unchanged between MeCP2 T58A KI mice and wild type littermates (n=5 for MeCP2 T158A mice and n=7 for wild-type littermate mice). Statistically significant difference was determined using the Mann-Whitney U test confirming significant reduction in withdrawal threshold in the ipsilateral paw *p* value **<0.01, ***<0.001.

Figure 5

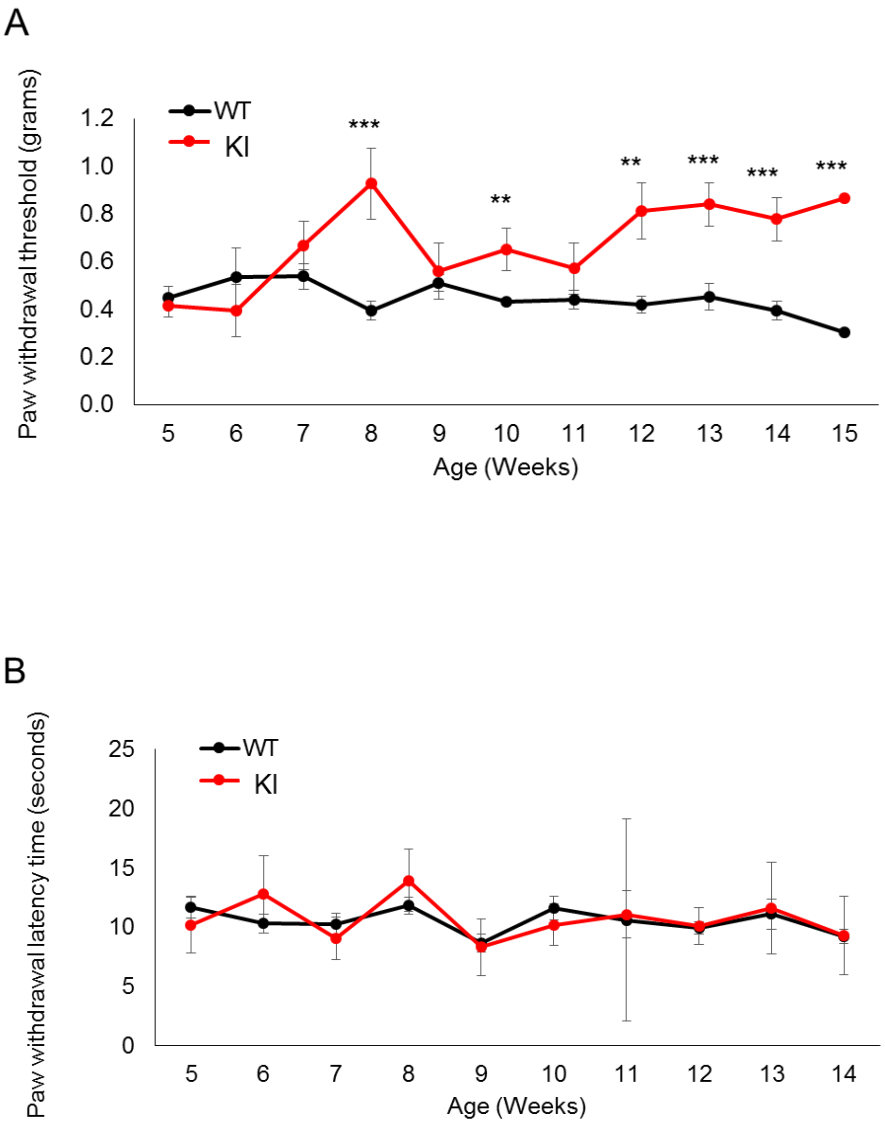
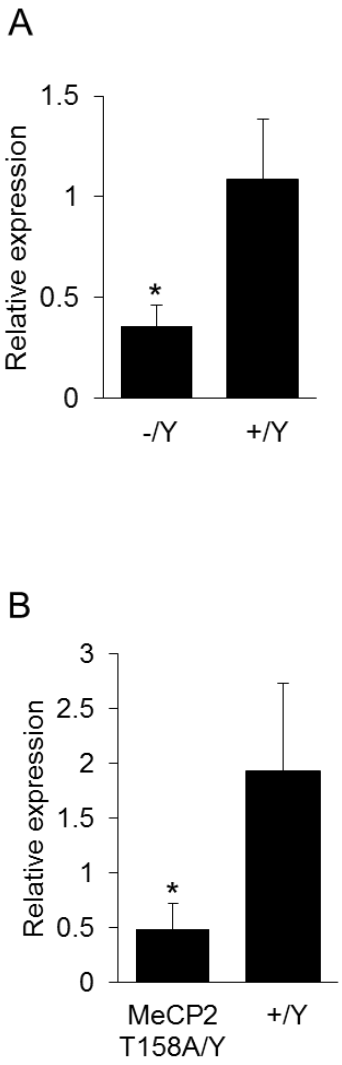
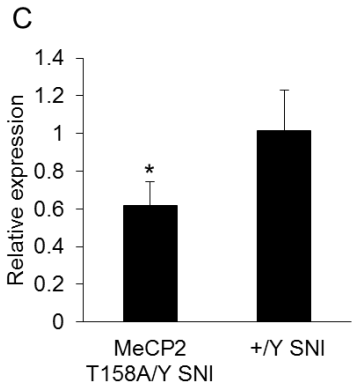

A
Paw withdrawal threshold (grams)
WT
KI
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
**
***
***
***
5
6
7
8
9
10
11
12
13
14
15
Age (Weeks)
B
Paw withdrawal latency time (seconds)
WT
KI
25
20
15
10
5
0
5
6
7
8
9
10
11
12
13
14
Age (Weeks)

Figure 6: Downregulation of *Bdnf* expression in Mecp2 mutant mice

A, DRG from *Mecp2*-null mice and ***B,*** MeCP2 T158A mice has significantly lower *Bdnf* mRNA compared to wild-type littermates, n=3. (n=6). ***C,*** Bdnf was also lower in the MeCP2 T158A mice compared to wild-type littermates after SNI surgery. Significance determined using Student's *t*-test, *p* value *<0.05.

Figure 6


A
Relative expression
1.5
1
0.5
0
*
-/Y
+/Y
B
Relative expression
3
2.5
2
1.5
1
0.5
0
*
MeCP2
T158A/Y
+/Y


C
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative expression
*
MeCP2 T158A/Y SNI
+/Y SNI

CHAPTER THREE: MeCP2 Mediated Regulation of Genes Involved in Pain in the Dorsal Root Ganglia

ABSTRACT

Methyl-CpG-binding protein 2 (MeCP2), a protein with affinity for methylated cytosines, is crucial for neuronal development and function. MeCP2 regulates gene expression through activation, repression and chromatin remodeling. Mutations in *MECP2* cause Rett syndrome (RTT) and these patients display impaired nociception. We observed an increase in MeCP2 expression in mouse dorsal root ganglia (DRG) after peripheral nerve injury. The functional implication of increased MeCP2 is largely unknown. To identify regions of the genome bound by MeCP2 in the DRG and the changes induced by nerve injury, MeCP2 ChIP-seq was performed. While the number of binding sites across the genome remained similar in the spared nerve injury (SNI) model and control, SNI induced the redistribution of MeCP2 to transcriptionally relevant regions. To determine how differential binding of MeCP2 can affect gene expression in the DRG, we investigated mmu-miR-126, a microRNA locus that had enriched MeCP2 binding in the SNI model. Repression of miR-126 expression and the ensuing downstream molecular changes in the DRG after nerve injury was confirmed using the SNI model, *Mecp2*-null mice and a neuronal cell line. Our study shows a regulatory role for MeCP2 in that, changes in global redistribution can result in direct and indirect modulation of gene expression in the DRG, thereby contributing to epigenetic alterations underlying nerve injury.

INTRODUCTION

Epigenetic modifications resulting from DNA methylation plays a critical role in cellular differentiation, development [32], and may contribute to disease including peripheral and central pain sensation and processing [59]. DNA methylation is mediated by DNA methyltransferase (DNMT) catalyzing the transfer of a methyl group onto the 5′ position of cytosine. MeCP2 can decipher methylation patterns across the genome before binding to methylated DNA [32], and can mediate downstream transcriptional changes of a large number of genes [31]. Depending on its interacting protein partners and target genes, MeCP2 can act either as an activator or repressor [94]. MeCP2 can also play a role in dampening genome-wide transcriptional noise in a DNA methylation-dependent manner [28]. Mutations in *MECP2* result in the neurodevelopmental disorder RTT [114]. Among the many symptoms associated with RTT, alterations in pain sensitivity are reported to be as high as 75% [63]. Reduced sensitivity has been observed in mouse models with RTT and autism-associated mutations [64-67]. The observations from RTT patients and MeCP2 mutant mice indicate that MeCP2 contributes either directly or indirectly to reduced pain sensitivity.

Our previous study has shown that MeCP2 expression was altered in mouse DRG following SNI. While we observed upregulation 4 weeks post-surgery [64], others have reported downregulation at earlier time points and in subsets of damaged neurons in the DRG [67, 71]. MeCP2 has previously been linked to inflammatory pain. Inflammatory stimulus was shown to increase phosphorylation

of MeCP2 in lamina I neurons in the dorsal horn, resulting in its dissociation from the genome, thereby alleviating repression of genes linked to pain [72]. MeCP2 has also been associated with central mechanisms of pain through regulation of a transcriptional repressor, histone dimethyltransferease G9a, resulting in increased expression of brain-derived neurotrophic factor [73].

MeCP2 is highly expressed in neurons and glia [28]. The dynamic expression of MeCP2 in the DRG after nerve injury [64] suggests a role for MeCP2 in pain modulation through transcriptional regulation. DRG is a major player responsible for conveying noxious stimuli from the periphery to the central nervous system. MeCP2 is predominantly a nuclear protein and cell bodies of nociceptive sensory neurons are located in DRG, with two axonal branches projecting to the periphery and to the dorsal horn of the spinal cord. Genome-wide studies investigating MeCP2 binding patterns have been conducted in neurons, astrocytes and different regions of the brain [28, 115-117]. To investigate the role of MeCP2 in mediating nociception, and to determine changes in MeCP2 binding patterns after nerve injury, we performed ChIP-seq in mouse DRG 4 weeks after SNI surgery. We observed a genome-wide redistribution of MeCP2 binding, and evaluated how changes in binding patterns directly and indirectly affect gene expression that contributes to the pathology of pain.

MATERIALS AND METHODS

Animal model of neuropathic pain

The care and use of all mice were approved by the Institutional Animal Care & Use Committee of Drexel University College of Medicine. The SNI model was generated using 8-week old C57BL/6 male mice (Taconic) as previously described [10, 102]. Briefly, mice were anesthetized with isoflurane during surgery. The common peroneal and tibial nerves of the left paw were ligated, 2-4mm of the nerve was sectioned and removed distal to ligation. Sham mice underwent the same surgical procedures as the SNI group without ligation and sectioning. Development of mechanical hypersensitivity was assessed using von Frey filaments [102]. L4, L5 and L6 DRG on the ipsilateral side of surgery were collected 4 weeks post SNI surgery at 12 weeks of age.

Mecp2-null mice

DRG from approximately 12 weeks old male hemizygous *Mecp2*-null (*Mecp2*$^{-/y}$) mice and wild type littermates (*Mecp2*$^{+/y}$) were collected for molecular characterization [77]. These mice do not have detectable *Mecp2* gene product and were used to study gene expression in the absence of MeCP2.

Chromatin immunoprecipitation (ChIP) of MeCP2

ChIP was performed using protocols adapted from Covaris truChIP chromatin shearing kit tissue SDS and Millipore magna ChIP G tissue kits. Ipsilateral L4, L5

and L6 DRG were collected 4-weeks after SNI or sham surgery. DRG was pooled from 10 mice per sample, in duplicates. Samples were washed 3 times in phosphate buffered saline (PBS) containing protease inhibitors (PI). Chromatin was crosslinked to DNA using 1% methanol-free formaldehyde and incubated at room temperature for 5 min with rotation. Samples were then incubated with Covaris quenching buffer for 5 min at room temperature with rotation to quench the formaldehyde crosslinking reaction. Samples were washed 3 times for 5 min with PBS containing PI. Tissue samples were resuspended in Covaris lysis buffer containing PI, incubated on ice for 10 min and homogenized by pipetting. After 10 min incubation on ice, nuclei were pelleted at 1700xg for 5 min at 4°C. Samples were washed using 1ml Covaris wash buffer, pellets resuspended in 130µl Covaris SDS shearing buffer containing PI and incubated on ice for 10 min. Chromatin was sheared using the Covaris M220. Insoluble material was pelleted by centrifugation at 10,000xg for 5 min. Five µl of the supernatant was removed for Input samples. To each sample, 20µl of Protein G magnetic beads and 5µl anti-MeCP2 antibody [78] or 5µl rabbit serum was added and incubated overnight at 4°C with rotation. Beads were pelleted with a magnetic separator, washed and resuspended in Millipore elution buffer containing proteinase K, followed by incubations at 62°C overnight, and 95°C for 10 min. Supernatant was used for DNA purification according to manufacturer's instructions.

Sequence Alignment and Filtering

Single-end 50 base reads were generated on the Applied Biosystems (AB) SOLiD 5500xl platform. Reads were mapped to the mm10 genome using AB LifeScope 2.5.1 software. Reads in each sample were filtered out based on their mapping quality, to eliminate poorly mapping reads and reads that may map to multiple places in the genome, and only reads MAPQ≥10 were used for peak calling and tag enrichment analysis. Samtools [118] merge was used to pool replicates for each IP condition, and samtools rmdup was used to remove duplicate reads so that only unique tags were used for peak calling.

Peak Calling

The HOMER (Hypergeometric Optimization of Motif EnRichment) suite [119] was used for peak calling on the sham and SNI IP sequence libraries, using respective sham and SNI input libraries as control. HOMER ChIP-seq tag directories were created from the set of unique mapped reads for each IP and input condition. HOMER peak calling (findPeaks) was performed for both narrow peaks (-style factor) and broad peaks (-style histone). Visual review of the sequence tags in the Integrated Genomics Viewer (IGV) [120] was used to decide whether narrow or broad peaks were a better fit for MeCP2 binding patterns. The set of broad peaks was selected as the better representation of MeCP2 binding and used for subsequent annotation and interpretation. HOMER was also used to identify differential binding between sham and SNI, using the getDifferentialPeaks tool,

with default settings to identify peaks with 4-fold more tags in one condition versus the other, with *p*-value less than 0.0001.

Annotation

The HOMER annotation database v5.4 for mouse was used in conjunction with the HOMER annotation tool (annotatePeaks) to identify the closest genomic feature to each peak, as well as enrichment statistics for genomic regions including promoters, exons, introns, intergenic regions, etc. Annotation was performed on the set of all sham peaks, all SNI peaks, as well as differentially bound peaks in sham and SNI. The HOMER annotatePeaks tool was also used in mRNA and miRNA mode to analyze tag enrichment and peak locations with respect to all annotated genes and miRNAs.

Cell culture and transfection

Neuro 2a cells obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO2. For monitoring changes in endogenous Dnmt1 and Vegfa expression, cells were transfected with precursor miR-126 plasmid (GeneCopoeia) using X-tremeGENE HP DNA transfection reagent (Roche) for 72 hours.

Quantitative RT-PCR

RNA was purified from the following samples: Neuro 2a cells, DRG collected from SNI model, sham control, *Mecp2*-null and wild-type littermate mice using the mirVana RNA isolation kit (Life technologies). cDNA synthesis and qRT-PCR for mRNA were performed as previously described [101]. The Assay ID for TaqMan primer probes used were Mm00599780_g1 (Dnmt1) and Mm00437306_m1 (Vegfa). Gapdh was used as a normalizer. cDNA synthesis for miR-126 and detection was conducted using TaqMan microRNA assay (Assay ID 00451, Applied Biosystems). U6 was used for normalization.

Western blot

Protein from Neuro 2a cells or DRG was isolated using radioimmunoprecipitation assay buffer (Thermo Scientific). For western blotting, 2µg protein lysate from DRG or 10µg from cell pellets were resolved by a 4-12% SDS–PAGE gel, transferred to nitrocellulose membrane. The membranes were probed with 1:500 dilution of Dnmt1 (D63A6) XP antibody #5032 (Cell Signaling) or 1:1000 Vegfa antibody (Abcam ab51745) overnight at 4°C. Chemiluminescence was detected using FluorChem M System (Protein Simple). The membrane was also probed with 1:1000 β tubulin (9F3) antibody #2128 (Cell Signaling) as a loading control. Quantification was done using UN-SCAN-IT software, Dnmt1 and Vegfa expression was normalized to β tubulin.

Immunocytochemistry

Neuro 2a cells grown on 12mm glass coverslips were transfected with miRNA precursor plasmids with GFP using X-tremeGENE HP DNA transfection reagent for 72 hours. Cells were fixed in 4% formaldehyde and blocked with 10% normal goat serum followed by a 3 hour incubation in 1:500 anti-Dnmt1 antibody (mentioned above). Anti-Rabbit-IgG Atto 647N secondary antibody (Sigma) was used for detection of Dnmt1. Coverslips were mounted using Vectashield mounting medium with DAPI (Vector Laboratories). Images were acquired using the 60x objective on the Olympus FV1000 confocal microscope and Fluoview FV10-ASW software. Transfected (GFP positive) and untransfected (GFP negative) cells were compared.

Intrathecal catheter implantation and miRNA injection

miRNA administration protocol was adapted from previous reports of intrathecal miRNA delivery [39]. To administer miRNA mimics, a polyurethane catheter (25G, 5.5cm long, SAI-infusion) was placed into the intrathecal space of the lumber L4-L5 vertebrae under isoflurane anesthesia. The catheter was stereotactically secured under the skin, and occluded between injections. A custom miRCURY (Exiqon) miR-126 mimic containing a 5’ cholesterol tag and 3’ fluorescein label was injected at 2nmol concentration with 4µl iFECT transfection reagent (Neuromics). A total of 6µl was delivered into the catheter connection juncture

using a 25G blunt end needle on a Hamilton syringe. The catheter was then flushed with 7µl sterile PBS to ensure miRNA reached the intrathecal space.

Bisulfite sequencing and analysis of CpG sites in miR-126 locus

To analyze the methylation status of CpG dinucleotides in the miR-126 locus, L4, L5 and L6 DRG were collected from 3 SNI or 3 sham control mice four weeks after surgery. Mouse genomic DNA was isolated using GenElute mammalian genomic DNA minipreps kit (Sigma Aldrich) and subjected to bisulfite conversion using the Epitect fast DNA bisulfite kit (Qiagen). The 72bp fragment of interest was amplified using the following primer pairs designed with MethPrimer software [121]; Forward: TATTTTGAAGAGGTTTTTGGAAGG,

Reverse: CCAAAACACACAACTAACTAAAAACAA.

Statistical methods

For statistical analysis, mean values with standard error are shown; *p* values were determined using Student's *t*-tests with paired or unpaired samples, significance was set at $p < 0.05$.

RESULTS

MeCP2–DNA binding profiles

To determine nerve injury induced changes in MeCP2-bound chromatin, we conducted ChIP-seq in the DRG from SNI model and sham control mice. Generation of SNI model, confirmation of hypersensitivity and DRG collection 4 weeks post-surgery have been previously described [64]. ChIP-seq was conducted on DRG pooled from 10 mice per sample. We determined that MeCP2 binding occurred broadly across the genome, as previously reported [28, 117]. Enriched binding was observed in promoter regions and gene bodies for both protein coding and noncoding genes in SNI and sham control DRG (Figure 1*A*-*C*). Comparison of MeCP2-bound sequences at single base resolution indicates distinct and common MeCP2 binding sites to regions encoding mRNAs in SNI and sham DRG samples (Figure 1*D*).

Genome-wide redistribution of MeCP2 binding in the DRG after nerve injury

Comparative analysis of enriched peaks in SNI versus sham was done to elucidate nerve injury induced differences in genome-wide MeCP2 binding. A redistribution of MeCP2 binding in SNI as indicated by increased enrichment within transcribed regions, is shown in Figure 2*A*-*B*. Table 1 shows ten genes with enriched MeCP2 binding in the promoter and in the gene bodies that have a four-fold increase or decrease (log2 fold change) between sham and SNI, with $p < 0.001$. Distinct

binding patterns of MeCP2 in SNI could therefore lead to alterations in the genome, which in turn can mediate gene expression changes ensuing nerve injury.

Enriched MeCP2 binding to miRNAs

We then investigated changes in MeCP2 binding to miRNAs after nerve injury. While miRNA-transcribed regions represent a much smaller fraction of the genome compared to protein-coding genes, our analysis highlighted a small number of miRNA loci with distinct MeCP2 binding pattern. A subsequent analysis of MeCP2-bound sequence tags indicated that miRNA regions were enriched for MeCP2 binding, and this enrichment was observed in the SNI model (Figure 3*A*). However, MeCP2 binding for several miRNA regions was detected in only one condition, sham or SNI, indicating different regulatory profiles in SNI versus sham. The miRNAs most enriched for MeCP2 binding, including 14 miRNAs with increased MeCP2 binding and 21 miRNAs with decreased binding in the SNI model relative to sham, are shown in Figure 3*B*. Of all the miRNAs with enriched MeCP2 binding, miR-126a and miR-126b have the largest fold enrichment in the SNI model. Therefore, we pursued further characterization of enriched MeCP2 binding to the miR-126 locus in the SNI model.

miR-126 locus has enriched MeCP2 binding in SNI

The peak profile for miR-126 locus from ChIP-seq shows enrichment for MeCP2 binding over input control and sham samples (Figure 4*A*). We observed an enrichment of MeCP2 binding to miR-126 locus after nerve injury, with virtually no

binding in the sham sample. This indicates that nerve injury induced an increase in MeCP2 binding to miR-126 locus and hence, we further explored the functional consequences of this binding in the context of nerve injury induced pain.

Methylation of miR-126 locus

Cytosine methylation of CpG dinucleotides is recognized as a crucial epigenetic modification. Since MeCP2 is a methyl DNA binding protein, we wanted to investigate whether increased MeCP2 binding to miR-126 locus observed in the DRG from SNI model, was due to changes in methylation pattern. Our analysis of the 72 bp sequence of premiR-126 locus revealed the presence of 6 CpG sites. To evaluate whether SNI surgery caused alterations in the methylation status of premiR-126 locus, we performed bisulfite sequencing of DRG genomic DNA obtained from SNI and sham control mice 4 weeks after surgery. Our analysis of the methylation status showed that all 6 CpG sites were methylated in both SNI and sham samples (Figure 4*B*). This identical methylation patterns in SNI and sham control mice indicates that nerve injury did not alter the methylation status of premiR-126 locus. Since there was an increase in MeCP2 bound to premiR-126 after nerve injury, we postulate that MeCP2 redistribution throughout the genome, and the specific enrichment at miR-126 could be induced by alterations in the availability of the genome for MeCP2 binding, after nerve injury.

miR-126 regulates expression of Dnmt1 and Vegfa in Neuro 2a cells

The Neuro 2a mouse neuroblastoma cell line was used to investigate regulation of target gene expression by miR-126. Previous studies have demonstrated direct binding of miR-126 to the 3'UTR of vascular endothelial growth factor (Vegfa) [118] and DNA methyltransferase 1 (Dnmt1) [122]. Neuro 2a cells were transfected with miR-126 precursor plasmid, or scrambled miRNA control, expressing GFP. We observed a decrease in mRNA (Figure 5*A*) and protein levels (Figure 5*B*) of Dnmt1 and Vegfa 72 hours after miR-126 transfection. We also show that miR-126 transfected cells have decreased Dnmt1 expression compared to untransfected cells (Figure 5*C*). Based on the decrease in both transcript and protein levels, we conclude that miR-126 regulates expression of both Dnmt1 and Vegfa through degradation of mRNA.

Repression of miR-126 and upregulation of its target genes in SNI model

We next sought to determine the consequence of increased MeCP2 binding to miR-126 locus in the DRG after nerve injury. There was a significant decrease of miR-126 in the DRG after SNI (Figure 6*A*). This indicates that increased binding of MeCP2 represses transcription of miR-126. We hypothesized that reduced levels of miR-126 would result in increased expression of miR-126 target genes in the DRG. An increase in *Dnmt1* and *Vegfa* transcripts were observed in the SNI model (Figure 6*B*-*C*). Western blot analysis confirmed an increase in Dnmt1 protein (Figure 6*D*). However, we did not observe significant changes in Vegfa protein in

the DRG (Figure 6*E*). Previous studies have shown that peripheral nerve injury leads to upregulation of Vegfa in accumulating neutrophils and macrophages in the peripheral nerve [123]. Vegfa is a secreted protein, therefore, protein expression in the DRG may not reflect miR-126 mediated regulation of Vegfa. Based on our *in vitro* data and reduced transcript levels of both target genes in the DRG after SNI, we conclude that downregulation of miR-126 levels resulting from MeCP2 binding, can contribute to the upregulation of Dnmt1 and Vegfa after nerve injury.

Expression of miR-126 and target genes Dnmt1 and Vegfa in DRG from *Mecp2*-null mice

To further confirm the regulatory role of MeCP2 on miR-126 expression, we obtained DRG from *Mecp2*-null mice. We assessed miR-126 levels in *Mecp2*-null mice and found that there was no significant differences compared to wild type littermates (Figure 7*A*). This finding, along with our observation from the SNI model indicates that MeCP2 does not regulate expression of miR-126 under naïve conditions. However, there was increased MeCP2 binding to miR-126 upon nerve injury, resulting in miR-126 repression. Since miR-126 is a negative regulator of Dnmt1 and Vegfa, we wanted to investigate if MeCP2 can also affect expression of these genes. Thus, we assessed mRNA levels of Dnmt1 and Vegfa in the DRG of *Mecp2*-null mice to determine the regulatory role of MeCP2. These mice do not have detectable levels of MeCP2. Our qPCR results showed a downregulation of Dnmt1 (Figure 7*B*) and Vegfa (Figure 7*C*) in the DRG in the absence of MeCP2,

suggesting that MeCP2 may directly or indirectly regulate the activation of these genes.

Overexpression of miR-126 *in vivo* did not alter pain threshold but decreased target gene expression

We observed regulation of Dnmt1 and Vegfa by miR-126 in Neuro 2a cells, and an inverse correlation in expression of miR-126 and its target genes in the DRG after nerve injury. To investigate whether upregulation of miR-126 can alter pain threshold in SNI model mice, we overexpressed miR-126 by intrathecal administration. An intrathecal catheter was implanted 4 weeks after SNI surgery, to allow for repeated administration of 2 nmol miR-126 or control miRNA. We did not observe a change in mechanical (Figure 8*A*) or thermal (data not shown) hypersensitivity in these mice, after overexpressing miR-126. With lack of efficacy in pain behavior studies, we wanted to confirm miR-126 delivery to the DRG, and investigate changes in Vegfa and Dnmt1 expression. Over 5 fold higher levels of miR-126 was detected in the DRG from mice that received miR-126 compared to those injected with control miRNA (Figure 8*B*), thus confirming uptake of miR-126 by the DRG. Additionally, delivery of miR-126 robustly downregulated *Dnmt1* and *Vegfa* mRNA in the DRG (Figure 8*C*). These results indicate that though the delivery of miR-126 decreased Dnmt1 and Vegfa expression in the DRG, this was not sufficient to reverse nerve injury induced mechanical and thermal hypersensitivity.

DISCUSSION

This study provides evidence indicating that peripheral nerve injury induces redistribution of MeCP2 binding in the DRG. MeCP2 is implicated in pain because of the abnormal thresholds observed in RTT patients [63]. Studies assessing the pain threshold of mice with aberrant MeCP2 expression, mimicking those observed in patients, have shown reduced sensitivity [64-67]. Collectively, these studies indicate that precise regulation of MeCP2 levels is crucial for normal pain thresholds. We had previously observed an upregulation of MeCP2 in the DRG 4 weeks after SNI. DRG neurons have been the focus of much research to identify molecular targets of pain neurotransmission because they represent primary sites for pain processing. Our data show that increased MeCP2 expression after peripheral nerve injury can induce differential binding of MeCP2 to the genome. This may play a crucial role in bringing about either direct or indirect global gene-regulatory changes associated with a chronic pain state.

MeCP2 was originally characterized for its high affinity for methylated cytosines that are followed by a guanine nucleotide (mCG) [32]. MeCP2 binds broadly throughout the genome in a DNA methylation-dependent manner. It is now known that MeCP2 can bind two alternatively methylated forms of DNA including methylated cytosine followed by a nucleotide other than guanine (mCH, where H = A, C or T) and hydroxymethylcytosine (hmC) [22, 23]. Both mCH and hmC are enriched in human and mouse brain but there are no reports to date on the status of these alternately methylated forms of DNA in the DRG. In general, mCH appears

to be a repressive mark that inhibits gene expression [22, 124]. The occurrence of hmC within gene bodies however, has been associated with active gene expression in neurons [23, 124]. Our ChIP-seq data suggests a genome-wide shift in the binding pattern of MeCP2 after nerve injury, including three thousand distinct mRNA encoding sites. There also seems to be a redistribution from binding at intergenic regions to an overall increase in binding to transcriptionally relevant regions and non-coding RNAs. MeCP2 quantification in postmitotic neurons showed that it is nearly as abundant as the histone octamer. It was suggested that genome-wide binding of MeCP2 in a DNA methylation-dependent manner may dampen transcriptional noise and MeCP2 may contribute to the basic structure of neuronal chromatin [28]. MeCP2 was shown to modulate transcription in a gene-length-dependent manner and preferential upregulation was observed for long genes enriched for methylated CpA dicnuleotides in brain from *Mecp2*-null mice [116]. Our tag enrichment analysis were more sensitive to binding changes in longer genes, simply because statistical power increases with tag count, and longer regions (gene bodies) have more changes of MeCP2 binding. Hence, our approach detected stronger binding differences in large genes. Thus binding of MeCP2 at mCG, mCH and hmC, along with the accompanying alteration in chromatin architecture can collectively play a role in regulating gene expression in mature neurons.

Neuropathic pain models use nerve injury to induce hyperalgesia and allodynia in rodents. Neurons in the DRG are surrounded by an envelope of

satellite glial cells and it is now well established that immune and glial cell responses alter neuronal function in peripheral and central nervous system [125]. Our ChIP-seq study was performed using whole DRG and thus the findings reported here is from a mixed cell population. Comparison of cell specific global DNA methylation along with gene expression data will enable comparative study to confirm if methylation induced changes in MeCP2 binding resulted in gene expression changes. Plasticity of DNA methylation was recently investigated in rat DRG 24 hours after spinal nerve ligation, another peripheral nerve injury model of neuropathic pain. Though there was widespread remodeling of DNA methylation, comparison of genome-wide methylation and RNA-seq analysis of promoter regions and gene bodies showed that variation of methylation was not tightly linked with differences in gene expression [126]. The impact of DNA methylation on transcription is context dependent because methylation has been shown to either inhibit or promote gene expression, depending on the location of the mark [127]. MeCP2 is an abundantly expressed protein that seems to bind throughout the genome, and therefore it is plausible that in addition to regulating specific target genes, MeCP2 may broadly affect genome-wide transcription.

MeCP2 alters gene expression through several mechanisms but MeCP2 function itself is regulated, by miRNAs and several post translational modifications including activity dependent phosphorylation [128]. MeCP2 also suppress miRNA biogenesis by directly binding to DiGeorge syndrome critical region 8 (DGCR8), a critical component of the nuclear miRNA-processing machinery [129]. MeCP2-

bound sequence tags indicated that miRNA regions were enriched for MeCP2 binding in the SNI model compared to sham control. It has been proposed that the functions of miRNAs are more pronounced under stress or disease states [130], and alterations in MeCP2 binding could be one of the mechanisms contributing changes in miRNA expression.

To investigate the impact of nerve injury induced redistribution, we studied miR-126, a miRNA with highest enrichment for MeCP2 binding post nerve injury. miR-126 is encoded within the intron of Egfl7 and highly expressed in brain [131]. A recent study demonstrated a role for miR-126 in reducing inflammation and improving the functional deficit after spinal cord injury. miR-126 decrease promoted angiogenesis and inflammation in the spinal cord after a weight induced contusion, and administration of miR-126 reduced locomotor deficit and tissue damage [132]. Nerve injury induced upregulation of MeCP2 and a decrease in miR-126 after SNI. There was no MeCP2 binding at the miR-126 locus in sham control mice. Furthermore, *Mecp2*-null mice and their wild type littermates have similar levels of miR-126. This indicates that nerve injury induces the enriched binding of MeCP2 to miR-126 locus, resulting in miR-126 repression. We also observed increased expression of Vegfa and Dnmt1 in the SNI model. Dnmt1 and Vegfa are two validated target genes of miR-126 [118, 122]. Both genes are implicated to have a role in peripheral pain mechanisms. Dnmt1 is necessary for maintenance methylation, gene regulation, and chromatin stability [133]. Dnmt1 mutations in humans contribute to hereditary sensory neuropathy [134], and

autonomic neuropathy [135]. Our current work and others have shown upregulation of Dnmt1 in the DRG after peripheral nerve injury [136]. Vegfa regulates angiogenesis in both development and various pathologies [137]. In the chronic constriction injury model of neuropathic pain, Vegfa and its receptor was upregulated in the DRG, and addition of anti-Vegf antibody increased thermal and mechanical withdrawal latency [119]. We observed increased *Vegfa* mRNA in the DRG after SNI. miR-126 regulation of these targets are further supported by our molecular studies. *Mecp2*-null mice had reduced levels of *Dnmt1* and *Vegfa*. Reduced transcript levels in *Mecp*2-null mice can be interpreted as MeCP2 having a role in activation of these genes. Since we did not observe a significant binding of MeCP2 to Dnmt1 and Vegfa, one of the indirect mechanisms contributing to their upregulation after SNI could be the repression of miR-126 by MeCP2.

DNA methylation is a very stable epigenetic modification. Since SNI and sham DRG had an identical methylation pattern at miR-126 locus, increased binding of MeCP2 to miR-126 can be considered to be independent of the methylation status, but dependent on nerve injury. Likely, nerve injury changes the chromatin architecture, allowing increased access for MeCP2 to bind miR-126 locus. Further studies are needed to explore the role of MeCP2 in altering chromatin availability.

Administration of miR-126 did not alter the pain threshold in mice after SNI, and this could be due to the severity of nerve injury used to generate chronic neuropathic pain. miRNAs are fine tuners of gene expression and the low pain

threshold generated by SNI could have been too severe for a single miRNA to reverse. Acting as a rheostat, the influence of miRNAs is often described as modest. As was reported for the studies in a spinal cord injury model [132], the role of miR-126 in reversing inflammation and functional deficits could not be captured in the behavioral assessments we employed. Future studies in less severe nerve injury or inflammatory pain models that may capture the involvement of Vegfa should be explored. Several miRNAs showed changes in MeCP2 binding after SNI. Administering a cocktail of these miRNAs, to regulate a larger number of downstream target genes, could potentially be beneficial in conferring analgesic effects.

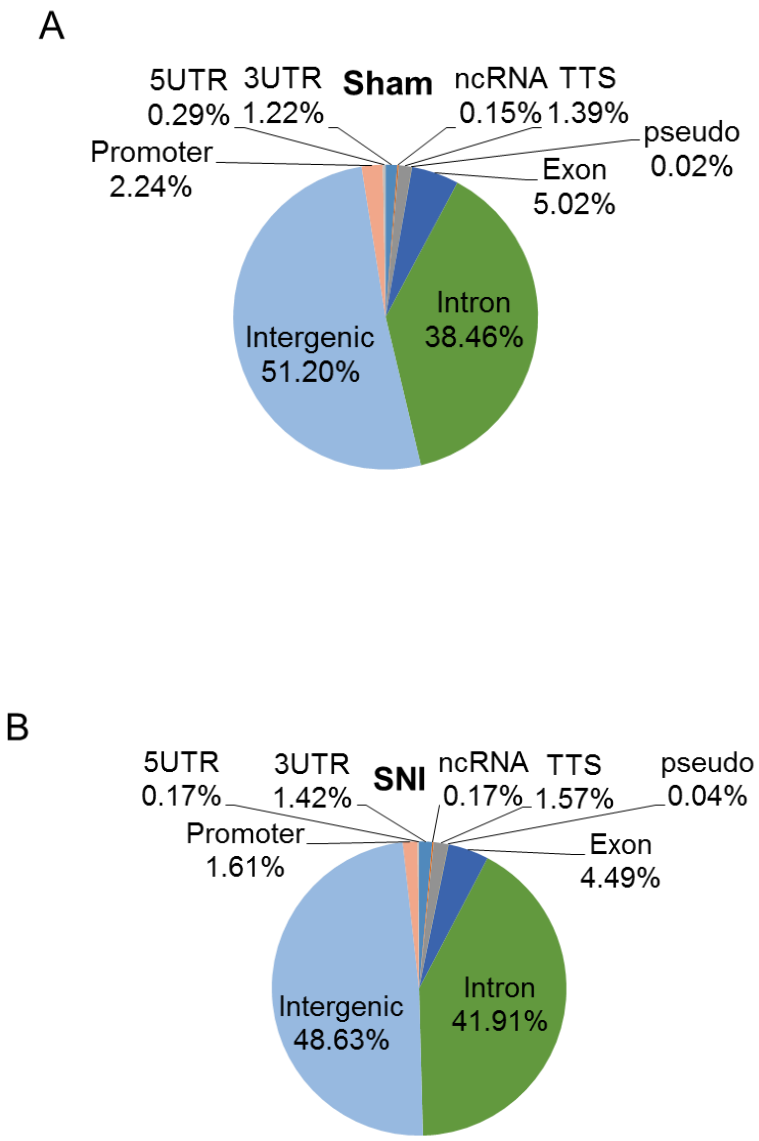
Collectively, these studies show that MeCP2 broadly binds chromatin, affecting numerous downstream targets. This study implicates a regulatory role for MeCP2, providing a molecular basis to better understand how epigenetic changes induce alterations in gene expression that occur in the DRG after nerve injury.

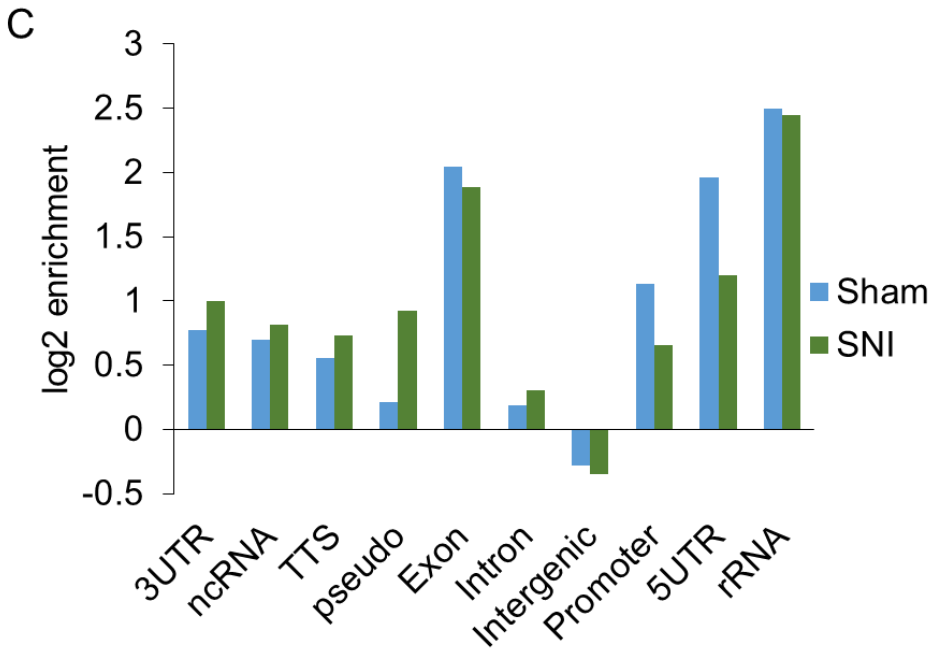
CHAPTER 3: Figure legends and Figures

Figure 1: Genome-wide MeCP2-DNA binding profile in DRG from SNI and sham control mice

A-B*,** Genomic distribution of peaks from ChIP-seq in the sham and SNI samples. ***C, Enrichment of MeCP2 binding to genomic regions in the SNI and sham models. ***D,*** Common and unique broad peaks identified in regions encoding mRNAs in SNI and sham. ChIP-seq data was generated from 2 IP samples each for SNI and sham. Each sample contained L4, L5, and L6 ipsilateral DRG obtained from 10 mice each, 4 weeks after surgery. 3UTR: 3'untranslated region, 5UTR: 5'untranslated region, ncRNA: noncoding RNA, Pseudo: pseudo genes, rRNA: ribosomal RNA, TTS: transcription termination sites.

Figure 1


A
Sham
5UTR 0.29%
3UTR 1.22%
ncRNA 0.15%
TTS 1.39%
pseudo 0.02%
Promoter 2.24%
Exon 5.02%
Intron 38.46%
Intergenic 51.20%
B
SNI
5UTR 0.17%
3UTR 1.42%
ncRNA 0.17%
TTS 1.57%
pseudo 0.04%
Promoter 1.61%
Exon 4.49%
Intron 41.91%
Intergenic 48.63%


C
log2 enrichment
3
2.5
2
1.5
1
0.5
0
-0.5
3UTR
ncRNA
TTS
pseudo
Exon
Intron
Intergenic
Promoter
5UTR
rRNA
Sham
SNI

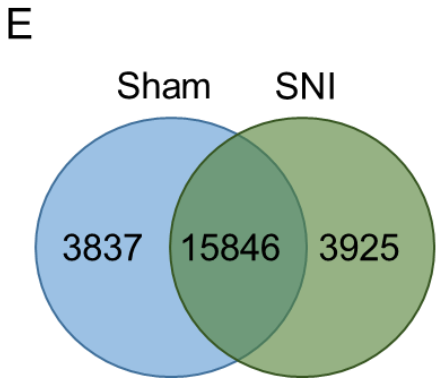
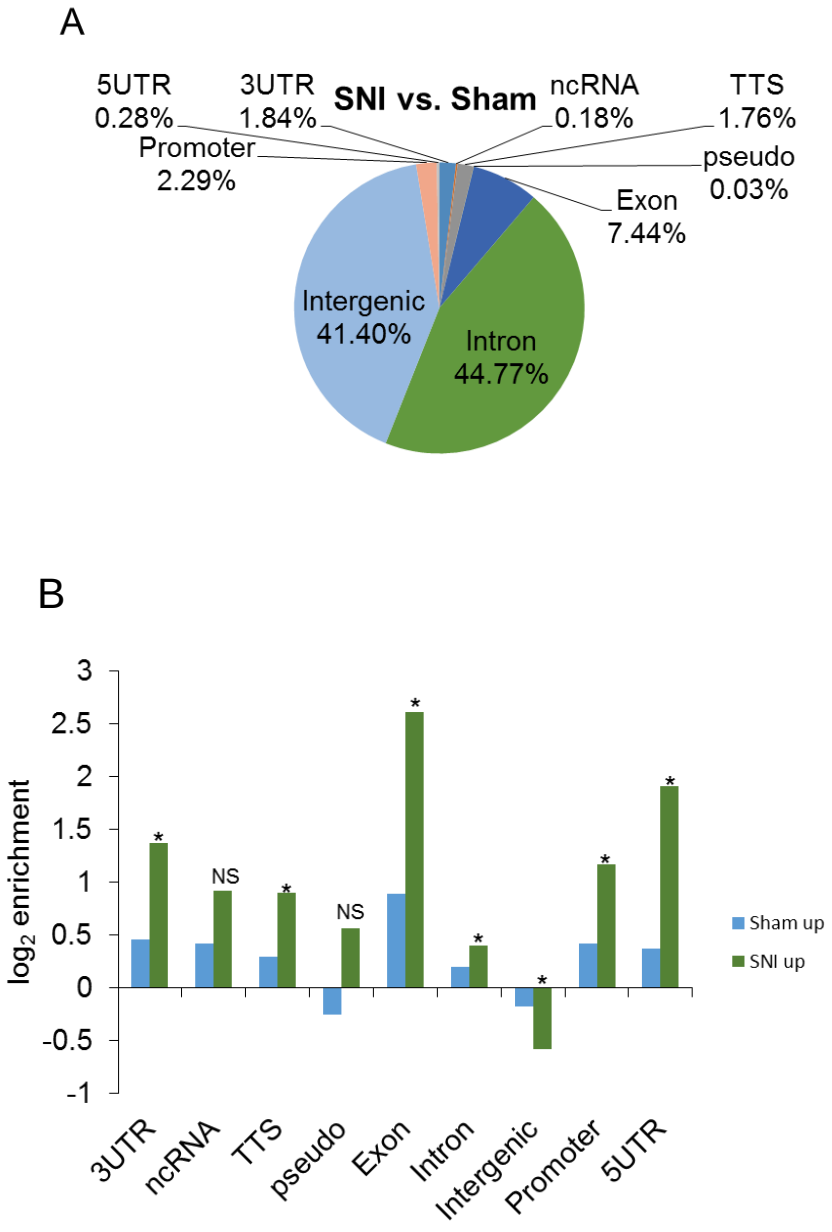

E
Sham
SNI
3837
15846
3925

Figure 2: Redistribution of MeCP2 binding in the DRG after nerve injury

A, Genome-wide distribution of enriched peaks representing putative MeCP2 binding sites that are at least 4 fold higher in the SNI model compared to sham control. ***B,*** Genomic redistribution of MeCP2 binding determined by enriched peaks in the SNI and sham model indicates increased MeCP2 binding to transcriptionally relevant regions and non-coding RNAs. 3UTR: 3'untranslated region, 5UTR: 5'untranslated region, ncRNA: noncoding RNA, Pseudo: pseudo genes, rRNA: ribosomal RNA, TTS: transcription termination sites; NS: not significant, * p value < 1×10^{-3}.

Figure 2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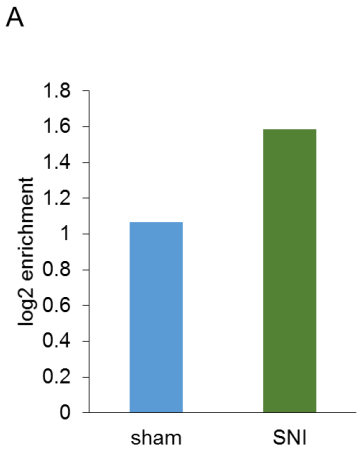
SNI vs. Sham
5UTR 0.28%
3UTR 1.84%
Promoter 2.29%
ncRNA 0.18%
TTS 1.76%
pseudo 0.03%
Exon 7.44%
Intergenic 41.40%
Intron 44.77%
B
log2 enrichment
3
2.5
2
1.5
1
0.5
0
-0.5
-1
NS
NS
*
3UTR
ncRNA
TTS
pseudo
Exon
Intron
Intergenic
Promoter
5UTR
Sham up
SNI up

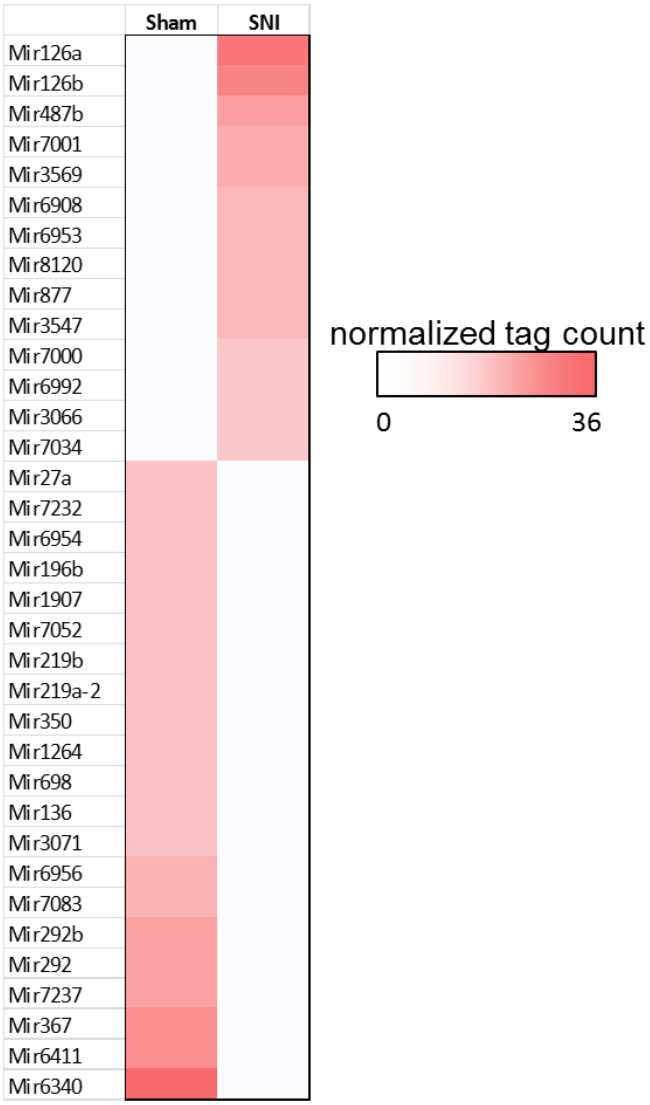
Figure 3: Enriched MeCP2 binding to miRNAs in the SNI model

A, miRNA tag enrichment is greater in the SNI model compared to sham control, suggesting altered MeCP2-mediated regulation of miRNAs after nerve injury, *p* value 1.2×10^{-3} ***B,*** miRNAs listed have at least 4 fold tag enrichment in the SNI or sham model. miR-126 was the miRNA with the highest tag enrichment in the SNI model.

Figure 3



B


Sham
SNI
Mir126a
Mir126b
Mir487b
Mir7001
Mir3569
Mir6908
Mir6953
Mir8120
Mir877
Mir3547
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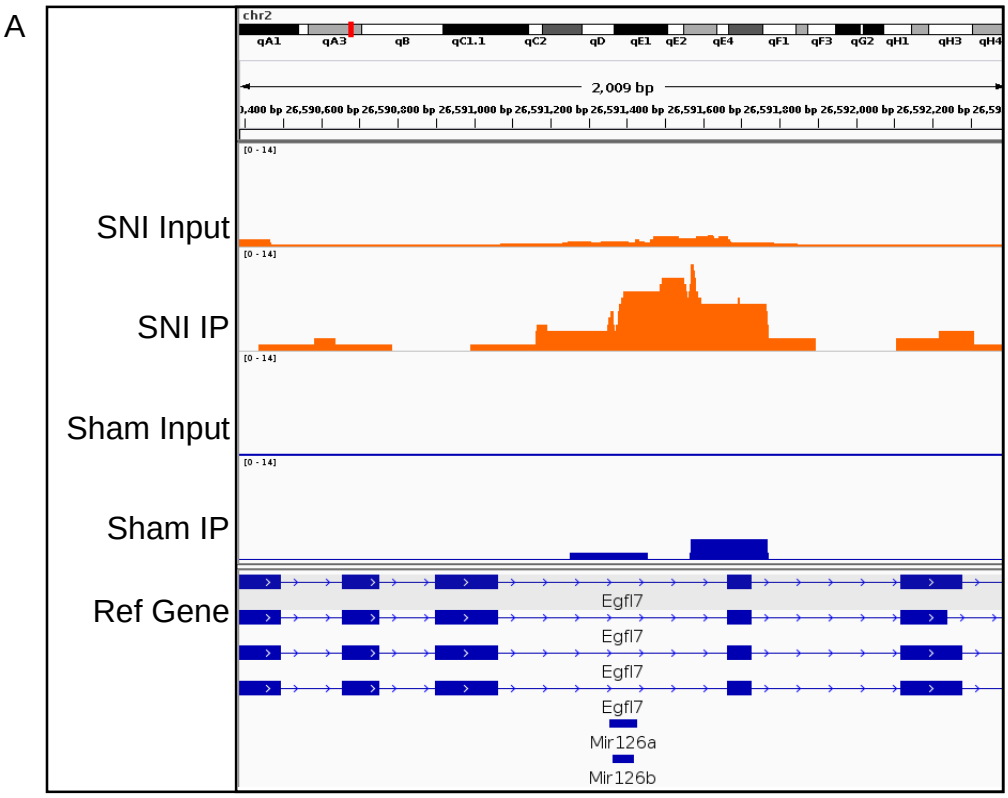
Mir6992
Mir3066
Mir7034
Mir27a
Mir7232
Mir6954
Mir196b
Mir1907
Mir7052
Mir219b
Mir219a-2
Mir350
Mir1264
Mir698
Mir136
Mir3071
Mir6956
Mir7083
Mir292b
Mir292
Mir7237
Mir367
Mir6411
Mir6340
normalized tag count
0
36

Figure 4: miR-126 locus has enriched MeCP2 binding in SNI model

A, MeCP2 peak profile from ChIP-seq at miR-126a/miR-126b shows enrichment of MeCP2 binding to miR-126 locus in the SNI model, and virtually no binding in the sham control. ***B,*** Histogram showing fold change enrichment of MeCP2 binding to miR-126 in the SNI model. ***C,*** CpG methylation of miR-126 locus was identical in SNI and sham control mice. Bisulfite sequencing of the 72 bp sequence of premiR-126 locus harboring 6 CpG sites using DRG genomic DNA obtained from SNI and sham control mice showed methylation of all CpG sites in both SNI and sham samples (*n*=3). Identical methylation pattern in SNI and sham control indicates that SNI does not alter the methylation pattern of premiR-126 locus.

Figure 4


A
chr2
qA1
qA3
qB
qC1.1
qC2
qD
qE1
qE2
qE4
qF1
qF3
qG2
qH1
qH3
qH4
2,009 bp
SNI Input
SNI IP
Sham Input
Sham IP
Ref Gene
Egfl7
Egfl7
Egfl7
Egfl7
Mir126a
Mir126b

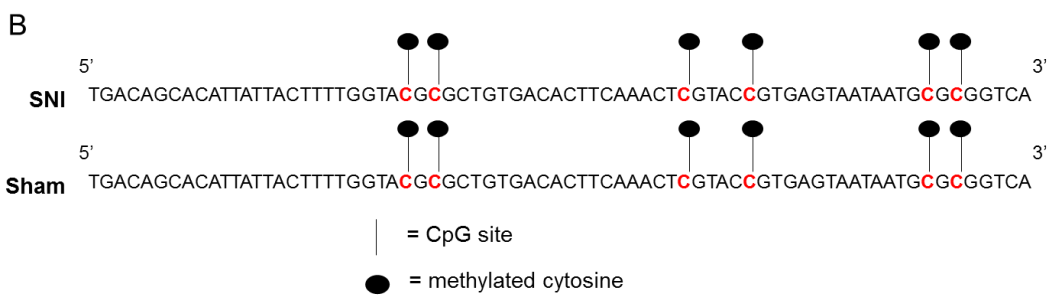
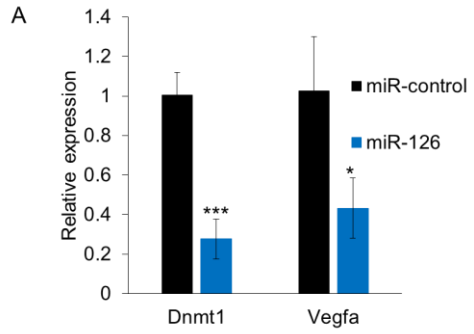
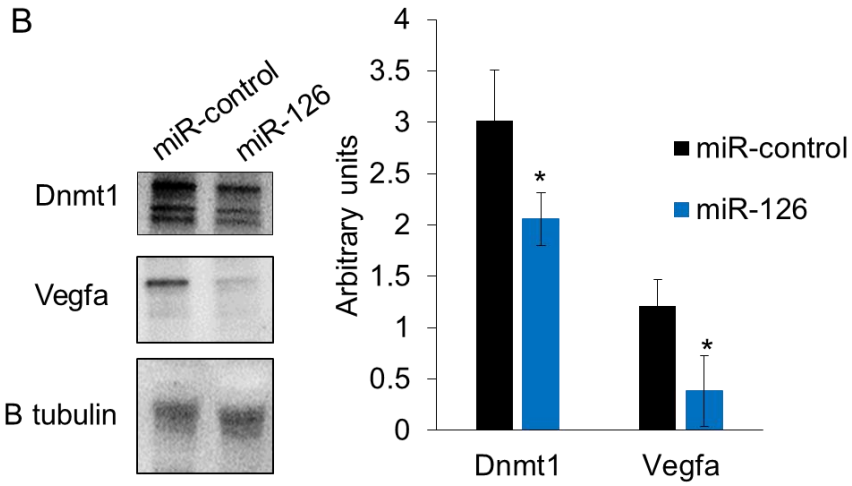

B
5'
3'
SNI TGACAGCACATTATTACTTTTGGTACGCGCTGTGACACTTCAAACTCGTACCGTGAGTAATAATGCGCGGTCA
5'
3'
Sham TGACAGCACATTATTACTTTTGGTACGCGCTGTGACACTTCAAACTCGTACCGTGAGTAATAATGCGCGGTCA
= CpG site
= methylated cytosine

Figure 5: miR-126 regulates expression of Dnmt1 and Vegfa *in vitro*

A, Relative expression of endogenous *Dnmt1* and *Vegfa* mRNA in Neuro 2a cells transfected with miR-126 *Gapdh* was used as a normalizer. ***B***, Representative western blot of Dnmt1 and Vegfa using lysate of Neuro 2a cells transfected with miR-126 precursor plasmid for 72 hours. Overexpression of miR-126 decreased mRNA and protein levels of Dnmt1 and Vegfa. β tubulin was used as the control. ***C***, Immunohistochemistry indicating transfection with miR-126 plasmid co-expressing GFP in Neuro 2a cells decreased Dnmt1 levels compared to untransfected cells 72 hours post transfection. Significance determined using Student's *t*-test, *p* value *<0.05, ***<0.001 (*n*=3 for all samples).

Figure 5


A
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
miR-control
miR-126
***
*
Dnmt1
Vegfa


B
miR-control
miR-126
Dnmt1
Vegfa
B tubulin
Arbitrary units
4
3.5
3
2.5
2
1.5
1
0.5
0
miR-control
miR-126
*
*
Dnmt1
Vegfa

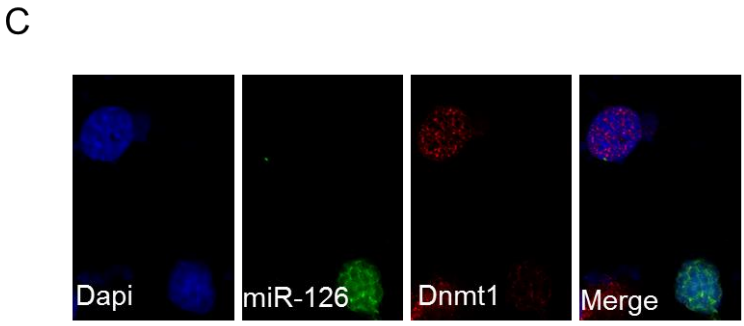
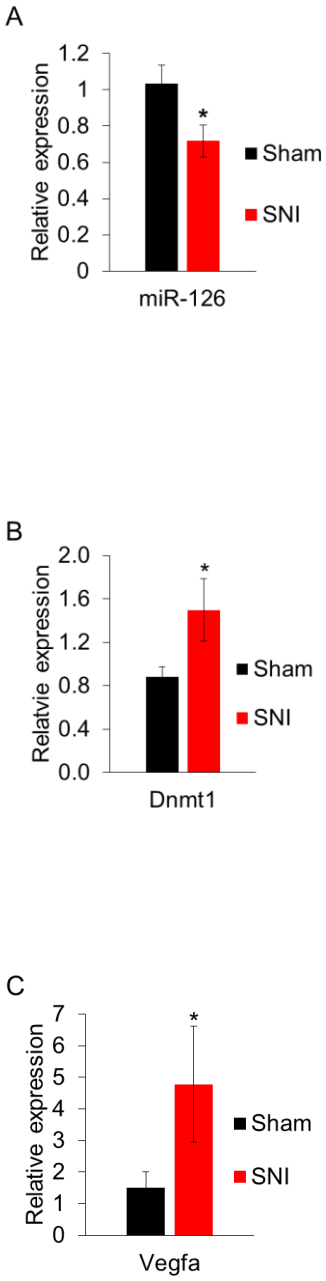
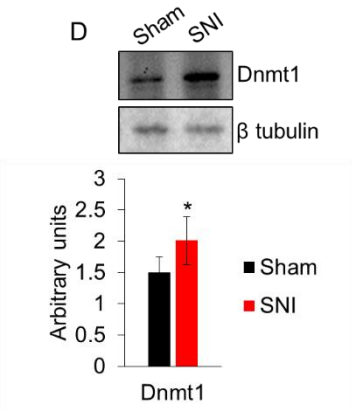

C
Dapi
miR-126
Dnmt1
Merge

Figure 6: Expression of miR-126 and its target genes Dnmt1 and Vegfa in the DRG after nerve injury

A, Relative expression of miR-126 determined by qPCR shows a reduction in miR-126 in SNI model compared to DRG from sham control. U6 was used for normalization (*n*=8 sham, *n*=7 SNI). ***B,*** Relative expression of *Dnmt1* mRNA and ***C,*** *Vegfa* transcripts showed an increase in the DRG after nerve injury compared to control (*n*=3). *Gapdh* was used as a normalizer. ***D,*** Representative western blot and quantification showed an increase of Dnmt1 protein in the DRG after nerve injury. ***E,*** Western blot and quantification showed Vegfa protein was not significantly different in DRG after nerve injury (*n*=3 from pooled samples, 3 DRG were pooled for each sample). Significance determined using Student's *t*-test, *p* value *<0.05.

Figure 6


A
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
*
Sham
SNI
miR-126
B
Relatvie expression
2.0
1.6
1.2
0.8
0.4
0.0
*
Sham
SNI
Dnmt1
C
Relative expression
7
6
5
4
3
2
1
0
*
Sham
SNI
Vegfa


D
Sham
SNI
Dnmt1
β tubulin
Arbitrary units
3
2.5
2
1.5
1
0.5
0
*
Sham
SNI
Dnmt1

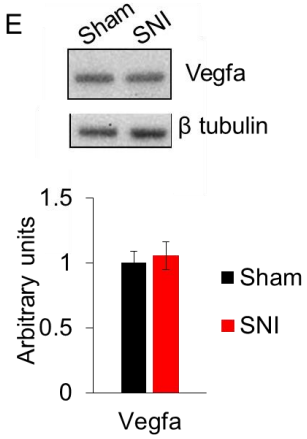

E
Sham
SNI
Vegfa
β tubulin
Arbitrary units
1.5
1
0.5
0
Sham
SNI
Vegfa

Figure 7: Expression of miR-126 and its target genes Dnmt1 and Vegfa in the DRG from *Mecp2*-null mouse

A, Relative expression of miR-126 in the DRG showed comparable expression in *Mecp2*-null and wild type littermates. U6 was used for normalization. ***B,*** *Dnmt1* and ***C,*** *Vegfa* expression were decreased in the DRG from *Mecp2*-null mouse, indicating MeCP2 has a role in regulating expression of Dnmt1 and Vegfa. *Gapdh* was used as a normalizer. Significance determined using Student's *t*-test, *p* value *<0.05, **<0.01 (*n*=3 for *Mecp2*-null (*-/y*) and wildtype littermate mice (*+/y*).

Figure 7

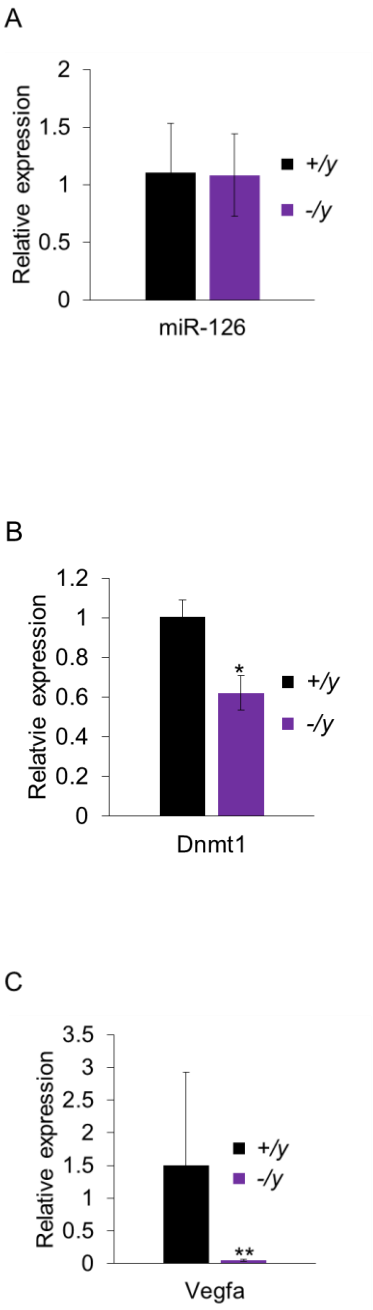
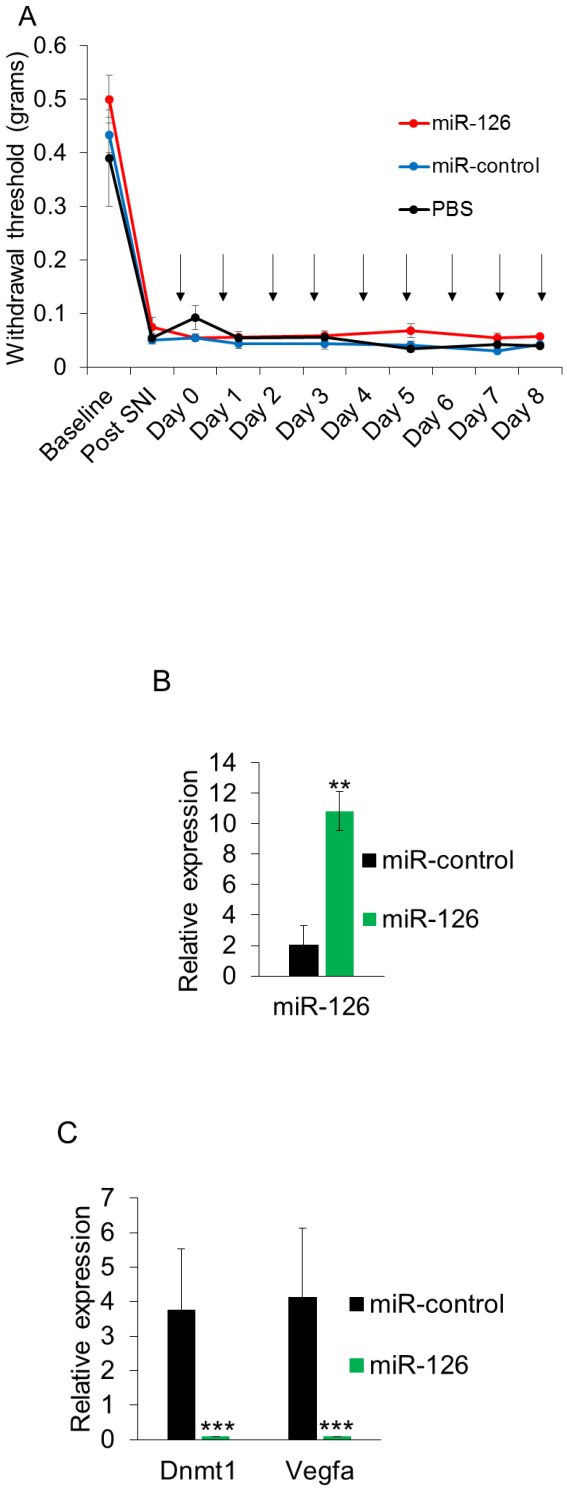

A
Relative expression
2
1.5
1
0.5
0
+/y
-/y
miR-126
B
Relatvie expression
1.2
1
0.8
0.6
0.4
0.2
0
*
+/y
-/y
Dnmt1
C
Relative expression
3.5
3
2.5
2
1.5
1
0.5
0
+/y
-/y
**
Vegfa

Figure 8: Administration of exogenous miR-126 decreased Dnmt1 and Vegfa expression *in vivo* but did not alter pain sensitivity

A, Mechanical sensitivity measured by von Frey filaments indicated that intrathecal delivery of miR-126 did not alter the paw withdrawal threshold in SNI model mice. Arrows indicate daily intrathecal injections with 2nmol miR-126 or control miRNA via catheter (*n*=5 for miR-126 and miR-control injected mice, *n*=3 for PBS injected mice). ***B,*** Confirmation of miR-126 delivery to DRG. A qPCR performed using DRG collected from mice injected with miR-126 or control miRNA showed an increase in miR-126 in mice that received miR-126 compared to miR-control injected mice indicating successful delivery. ***C,*** Relative expression of *Dnmt1* and *Vegfa* mRNA in the DRG of miR-126 and control injected mice. Increased miR-126 decreased the expression of endogenous *Dnmt1* and *Vegfa* compared to control miRNA injected mice. Significance determined using Student's *t*-test, *p* value, **<0.01, ***<0.001. (*n*=5 for miR-126 and miR-control injected mice, *n*=3 for PBS injected mice). ***D,*** Schematic representation of nerve injury induced alterations of MeCP2 binding and downstream gene expression changes. SNI induced enriched binding of MeCP2 to miR-126 locus resulting in repression of miR-126. Lower miR-126 leads to increased expression of its two target genes Vegfa and Dnmt1. Vegfa could contribute to the progression of pain pathology by modulating angiogenesis and neuro-inflammation. Dnmt1 propagate established methylation patterns during cellular division by recognizing and copying parent strand methylation at symmetrical CG dinucleotides to the newly synthesized daughter strand and its

increase after SNI can alter or maintain methylation, and may contribute to gene expression changes ensuing SNI.

Figure 8


A
Withdrawal threshold (grams)
0.6
0.5
0.4
0.3
0.2
0.1
0
miR-126
miR-control
PBS
Baseline
Post SNI
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
B
Relative expression
14
12
10
8
6
4
2
0
**
miR-control
miR-126
miR-126
C
Relative expression
7
6
5
4
3
2
1
0
***
***
miR-control
miR-126
Dnmt1
Vegfa

D

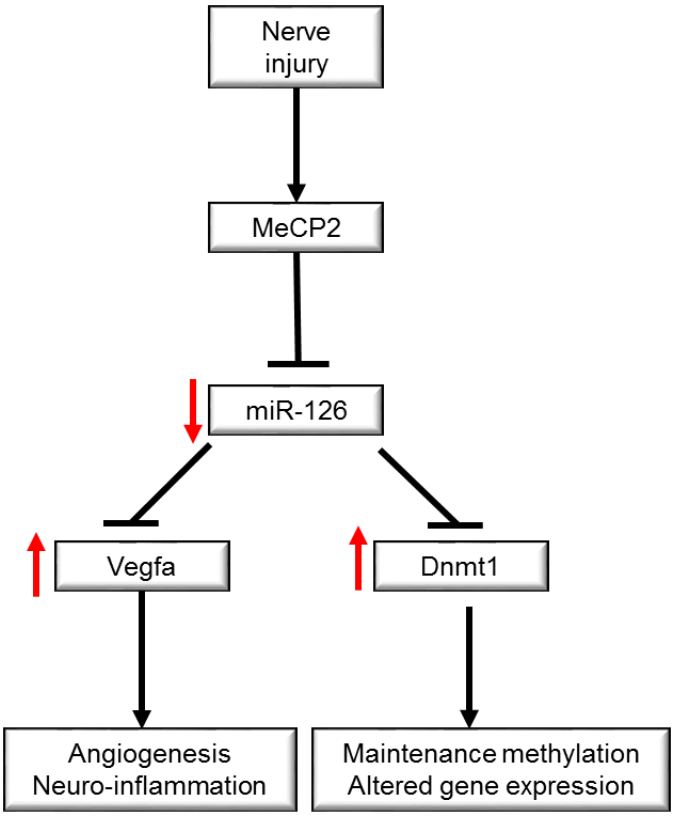

Nerve injury
MeCP2
miR-126
Vegfa
Dnmt1
Angiogenesis
Neuro-inflammation
Maintenance methylation
Altered gene expression

Table 1: Top ten MeCP2 enriched binding sites in the SNI model

Top ten MeCP2 enriched promoters and genes identified by ChIP-seq in the DRG from the SNI model compared to sham control.

Table 1

Promoters with enriched MeCP2 binding			Tag count		
Nearest Refseq	**Gene Name**	**Gene Description**	**sham**	**SNI**	***p* value**
NM_021478	Tulp1	tubby like protein 1	0	30	2.85E-06
NM_027398	Kcnip1	Kv channel-interacting protein 1	0	19	0.000195
NM_170757	Ccdc186	coiled-coil domain containing 186	0	16	0.000629
NM_001034898	Ms4a15	membrane-spanning 4-domains, subfamily A, member 15	0	25	1.92E-05
NR_033168	Snora28	small nucleolar RNA, H/ACA box 28	0	26	1.31E-05
NM_001113379	Lrrc32	leucine rich repeat containing 32	0	25	1.92E-05
NM_011305	Rxra	retinoid X receptor alpha	0	15	0.000932
NM_018820	Sertad1	SERTA domain containing 1	0	17	0.000425
NM_001011525	Olfr1415	olfactory receptor 1415	0	16	0.000629
NM_133759	Zbtb3	zinc finger and BTB domain containing 3	0	25	1.92E-05
Gene bodies with enriched MeCP2 binding					
NM_001001807	Olfr279	olfactory receptor 279	0	21	8.97E-05
NM_011540	Tcap	titin-cap	0	16	0.000629
NM_011971	Psmb3	proteasome (prosome, macropain) subunit, beta type 3	0	19	0.000195
NM_198414	Paqr9	progestin and adipoQ receptor family member IX	0	20	0.000132
NM_133668	Slc25a3	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 3	0	15	0.000932
NM_001160356	Uqcc3	ubiquinol-cytochrome c reductase complex assembly factor 3	0	15	0.000932
NM_001190356	Gm4832	predicted gene 4832	0	15	0.000932
NM_133759	Zbtb3	zinc finger and BTB domain containing 3	0	25	1.92E-05
NM_009296	Supt4a	suppressor of Ty 4A	0	20	0.000132
NM_009756	Bmp10	bone morphogenetic protein 10	0	18	0.000288

CHAPTER FOUR: Summary and Future Directions

OVERALL SUMMARY

This thesis was aimed at obtaining a fresh perspective and understanding of the molecular mechanisms underlying chronic pain mediated by epigenetic regulation. We investigated the mechanism and functional consequences resulting from upregulation of methyl-CpG-binding protein 2 (MeCP2) in the dorsal root ganglia (DRG), in mediating nerve injury induced chronic pain.

MeCP2 is critical for neuronal growth and function and dysregulation leads to neurological disorders, therefore, it is important to identify mechanisms of regulating MeCP2. Our previous study found 12 miRNAs predicted to target MeCP2 that were downregulated after nerve injury, and here, we further explored the regulation of MeCP2 by miRNAs. First, we demonstrate *in vitro* that miR-301 and miR-19a bind to the 3'untranslated region (UTR) of MeCP2 and regulate expression through translational repression. Downregulation of these miRNAs in the DRG is linked with upregulation of MeCP2 after nerve injury. We also showed that repression of MeCP2 by miRNAs affects MeCP2 mediated regulation of brain derived neurotrophic factor (Bdnf). MeCP2 has been shown to regulate Bdnf expression, and disruption of Bdnf is observed in *Mecp2*-null mice [31]. We found that *Mecp2*-null and MeCP2 T158A mice have decreased Bdnf in the DRG. MeCP2 T158A mice also have reduced sensitivity to an acute mechanical stimulus. Bdnf is an important modulator of sensory neurotransmission, with most of its biological effects mediated by the TrkB receptor. Signaling through this receptor contributes to the induction and maintenance of pain [106]. This could in

part explain why MeCP2 mutant mice and Rett syndrome (RTT) patients have decreased sensitivity. Together, these studies demonstrate that miRNA-mediated regulation of MeCP2 in the DRG and concomitant regulation of Bdnf could be important in pain sensation, and we demonstrate how a mutation in MeCP2 that leads to RTT causes reduced mechanical sensitivity.

The next set of studies presented here investigated the relationship between increased levels of MeCP2, and how this alters gene expression in the DRG after nerve injury. ChIP sequencing has been performed in the brain and subpopulations of neuronal and glial cells, however, this is the first MeCP2 ChIP-seq study conducted in the DRG. Additionally, our genome-wide comparative analysis of the MeCP2 binding profile after nerve injury identified differentially bound regions of the genome. This study addresses the role of MeCP2 in pain, and furthermore, our investigation of miR-126 demonstrates how changes in MeCP2 binding after nerve injury can have direct and indirect effects on regulation of genes.

We predicted that upregulation of MeCP2 leads to increased MeCP2 bound chromatin. Surprisingly, we found that total binding sites were similar between the spared nerve injury (SNI) and sham samples. However, MeCP2 binding was redistributed from intergenic regions to transcriptionally relevant regions and non-coding RNAs, and over 3,000 mRNAs were uniquely bound by MeCP2 in the SNI model. This data collectively suggests that a large number of genes are subjected to differential regulation by MeCP2. We additionally measured mRNA levels of

seven genes with enriched MeCP2 binding that are linked to pain, and found they were trending toward or were significantly upregulated in the SNI model (Figure 1). It has been previously described that MeCP2 has a role in regulating transcriptional noise [28], and our observation of a broad alteration in gene expression suggests that nerve injury-induced redistribution of MeCP2 could have a role in modulating transcriptional noise that leads to changes in gene expression. Correlating our MeCP2 genome-wide binding data with transcriptional profiling of the DRG would help elucidate if the redistribution of MeCP2 leads to altered gene expression.

We explored the functional outcomes of enriched MeCP2 binding to miRNA loci by investigating miR-126. MeCP2 was not bound to the miR-126 locus in sham mice, but was highly enriched after nerve injury. Our data indicate that enriched binding of MeCP2 represses miR-126 and results in increased expression of confirmed targets, DNA methyl transferase 1 (Dnmt1) and vascular endothelial growth factor (Vegfa). In order to determine the role of MeCP2 regulation of miR-126 in a naïve state, we measured expression in the DRG from *Mecp2*-null mice and found no changes in miR-126 levels compared to wild type littermates. We concluded that MeCP2 does not play a role in regulation of miR-126 until binding is induced by nerve injury.

The mechanism by which MeCP2 mediated regulation of miR-126 affects pain was evaluated by exploring validated targets of miR-126, Dnmt1 and Vegfa. Both genes are implicated to have a role in peripheral pain mechanisms. Dnmt1 is

necessary for maintenance methylation, gene regulation, and chromatin stability [133]. Mutations in *DNMT1* in humans contribute to hereditary sensory neuropathy [134], and autonomic neuropathy [135]. Our current work and others has shown upregulation of Dnmt1 in the DRG after peripheral nerve injury [136]. Vegfa regulates angiogenesis in both development and various pathologies [137]. In the chronic constriction injury model of neuropathic pain, Vegfa and its receptor were upregulated in the DRG, and administration of anti-Vegf antibody increased thermal and mechanical withdrawal latency [138]. In this study, we observed increased Vegfa mRNA in the DRG after SNI, and confirm miR-126 regulation of Dnmt1 and Vegfa with both *in vitro* and *in vivo* overexpression studies.

These experiments demonstrate that MeCP2 can indirectly regulate expression of Dnmt1 and Vegfa, through miR-126. To evaluate the direct regulatory role of MeCP2 on these targets, we measured transcripts in the DRG of *Mecp2*-null mice. Both Dnmt1 and Vegfa were significantly downregulated. We therefore conclude that activation of Dnmt1 and Vegfa is either directly or indirectly dependent upon MeCP2, while miR-126 is responsible for repression (Figure 2).

We investigated the methylation changes occurring directly on the miR-126 locus where MeCP2 binding was detected. Based on the enrichment of MeCP2 after nerve injury, we also expected increased methylation, particularly since the miR-126 locus has a high incidence of CpG sites and therefore could be subjected to methylation changes [139]. We found that CpG sites had an identical methylation pattern in both SNI and sham DRG. Nerve injury likely changes

chromatin structure, allowing increased access for MeCP2 to bind miR-126. Further studies are required to explore how nerve injury alters nucleosome positioning and how redistribution of MeCP2 induces or responds to these alterations.

Intrathecal injection of miR-126 confirmed the molecular mechanism in that, overexpression of miR-126 resulted in downregulation of Dnmt1 and Vegfa. However, exogenous miR-126 did not reverse mechanical allodynia or hyperalgesia associated with the SNI model. Likely, a single miRNA with targets that are involved in gene regulation and inflammation would not be therapeutic in this severe neuropathic pain model. It is also possible that this mechanism has temporal limitations; repressing Dnmt1 and Vegfa may be more efficacious at an earlier time point, or even prior to SNI surgery. Another group saw significant alteration of mechanical allodynia by administering miR-124 immediately after SNI surgery [50]. It may be difficult to reverse a behavioral phenotype by miRNA administration 4 weeks post-surgery, when molecular modifications have significantly progressed.

Dnmt1 is responsible for methylation of hemimethylated CpG sites after cell division. Increased levels of Dnmt1 after nerve injury could have effects on the global methylation pattern in the DRG. This may be particularly evident in actively dividing cells, such as astrocytes and glia. The resulting methylation changes could directly repress gene expression, or could influence MeCP2 binding patterns. Therefore, it would be interesting to evaluate global methylation changes in

specific populations of cells in the DRG. Further studies determining how MeCP2 binding correlates with changes in methylation pattern, and the global effects on transcriptional profile in the DRG should be pursued to explore this concept.

This work demonstrates the role of MeCP2 in pain in a peripheral nerve injury model. Research on MeCP2 has mostly been focused on the central nervous system. Here, we demonstrate that MeCP2 can play a significant role in the peripheral nervous system. We initiated studies by assessing miRNA-mediated regulation of MeCP2, followed by a genome-wide ChIP-seq study and an in-depth analysis of miR-126 in the DRG. Our global study served to identify differentially bound genes after nerve injury, and both genome-wide and gene specific follow up studies will be beneficial to further elucidate the role of MePC2 in mediating pain. While our goal was to link MeCP2 to pain, this work is also relevant to the RTT community. RTT patients suffer from physical disabilities, reduced motor function, and altered pain sensitivity. This implicates a role for MeCP2 in the peripheral nervous system. Studies conducted in the periphery could contribute to the growing body of work investigating the many physical abnormalities associated with RTT.

FUTURE DIRECTIONS

Further investigation of the role of MeCP2 in pain modulation is important in understanding how epigenetic regulators can affect gene expression related to pain sensation and processing. This study is the first step in evaluating how MeCP2 effects expression of genes in the DRG.

After conducting genome-wide comparative analysis of MeCP2 binding in the DRG, we found 14 miRNAs that were highly enriched for MeCP2 binding. In this study we focused on miR-126, however it would be interesting to expand the investigation to other miRNAs in this list, for example miR-487b and miR-877. miR-487b is predicted to regulate expression of GRM3, a metabotropic glutamate receptor with a role in pain, and miR-877 is predicted to regulate β-tubulin [41], which could result in polymerization changes associated with neuropathic pain. Increased binding of MeCP2 could lead to differential expression of these regulatory miRNAs and may contribute to the progression of chronic pain through regulation of their predicted targets. Further studies including confirmation of target binding, repression of target expression, and *in vivo* administration followed by behavioral assessment could be pursued. There are also 21 miRNAs that have reduced MeCP2 binding in the SNI model compared to sham. This could lead to altered miRNA levels after nerve injury and ultimately affect target expression. miR-27a has reduced MeCP2 binding in the SNI model and regulates transcription factors RUNX1 [140] and FOXO1 [141]. RUNX1 is expressed in non peptidergic nociceptors and regulates expression of several ion channels and mu opioid

receptors [142].These transcription factors affect a large number of downstream genes and further investigation of their targets could provide a mechanistic link to pain.

miRNAs are fine tuners of gene expression and individually can have relatively small effects. It would be interesting to investigate changes in pain sensitivity after administration of a cocktail of miRNAs instead of individual miRNAs. Each miRNA has multiple targets, therefore administration of several miRNAs would have diverse molecular effects and could broaden the therapeutic potential. One issue with this approach is that regulation of multiple molecular targets could heighten the potential for side effects. This would have to be evaluated on an individual miRNA basis. Advances in miRNA administration to specific target cells and tissue will be critical in increasing the therapeutic potential of miRNAs.

To better understand regulation of miRNAs, studies can be conducted to identify methylation pattern and enrichment of MeCP2 binding within the promoter of miRNAs and genes. Promoter regions have not been mapped for most miRNAs; there are often multiple transcriptional start sites [12], and promoters can be intronic, exonic, or shared with the promoter of the host gene [13]. The promoter for miR-126 has not been defined, however, it could be shared with one of the promoters of its harboring gene, Egfl7, as expression of the gene and miRNA seem to be proportional [139]. This T2 promoter also contains a CpG island, and typically, methylation at promoters is associated with gene silencing. Evaluating

the methylation status of this putative promoter may help us further understand how nerve injury and enriched MeCP2 binding repress miR-126 expression in the DRG.

There are many loci enriched for MeCP2 after SNI surgery. We measured expression levels of selected genes with potential roles in pain (Figure 1). Of the seven genes we measured, all were upregulated in the DRG after nerve injury. Further evaluation of the direct or indirect role of MeCP2 in regulation of these individual genes and their contribution to pain is necessary to better understand the role of MeCP2 in pain in the peripheral nervous system. Our comparative analysis focused on genes with enriched binding in the SNI model compared to sham. However, the reciprocal approach should also be taken to investigate genes with less MeCP2 binding in SNI compared to sham. This would reveal a new list of differentially bound genes that could have a role in mediating pain.

Finally, we found that MeCP2 levels are decreased in the dorsal horn of the spinal cord after nerve injury and we conducted ChIP-seq on these samples. While this work is not included in the thesis presented here, we will conduct a comparative analysis and evaluate genes with enriched binding and altered expression in the SNI model. This study will further elucidate how an epigenetic regulator can influence gene expression relevant to pain.

CHAPTER: Figure Legends and Figures

Figure 1: Expression of genes with enriched MeCP2 binding in the SNI model

qPCR was used to determine transcript levels of genes that had comparatively enriched MeCP2 binding in the SNI model versus sham model. These genes were selected from a larger list as having potential roles in pain. Significance determined by Student's *t*-test, *p* value *<0.05

Figure 1

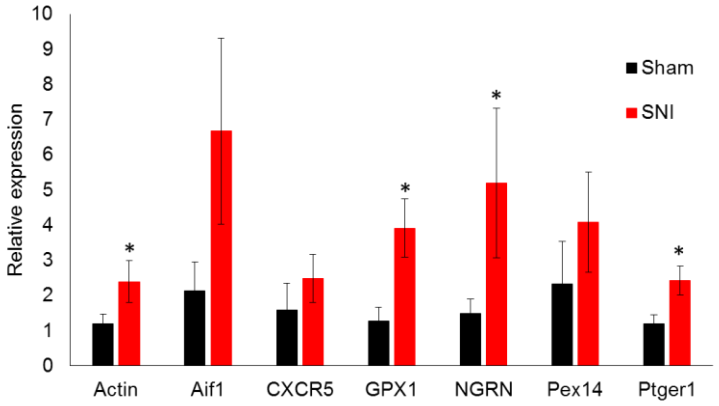
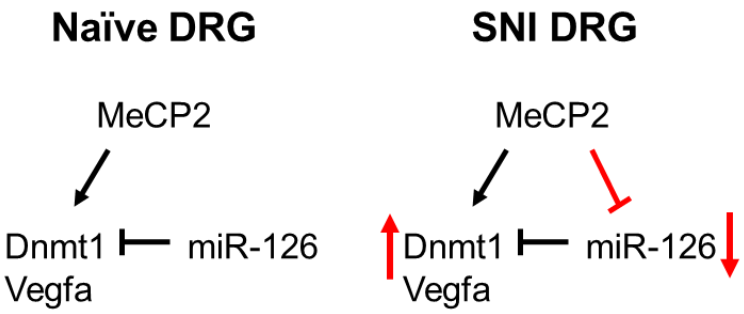

Relative expression
10
9
8
7
6
5
4
3
2
1
0
Sham
SNI
*
*
*
*
Actin
Aif1
CXCR5
GPX1
NGRN
Pex14
Ptger1

Figure 2: Schematic representation of Dnmt1 and Vegfa regulation

We propose that Dnmt1 and Vegfa is dependent upon MeCP2 for activation and miR-126 for repression. This homeostasis is disrupted in the SNI model. MeCP2 repression of miR-126 leads to upregulation of these targets.

Figure 2


Naïve DRG
MeCP2
Dnmt1
miR-126
Vegfa
SNI DRG
MeCP2
Dnmt1
miR-126
Vegfa

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